PCT WORLD INTELLECTUAL
International

WO 9603504A1

INTERNATIONAL APPLICATION PUBLISHED U

(51) International Patent Classification 6 :
C12N 15/12, 15/85, C07K 14/705, C12N 5/10, C12Q 1/68, C07K 16/28, A01K 67/027, C12Q 1/00

A1

(11) International Publication Number: WO 96/03504

(43) International Publication Date: 8 February 1996 (08.02.96)

(21) International Application Number: PCT/US95/09104

(22) International Filing Date: 18 July 1995 (18.07.95)

(30) Priority Data:
08/278,635 21 July 1994 (21.07.94) US

(71) Applicant: THE SALK INSTITUTE FOR BIOLOGICAL STUDIES [US/US]; 10010 North Torrey Pines Road, La Jolla, CA 92037 (US).

(72) Inventors: ELGOYHEN, Ana, Belen; 1844 Camino Del Mar #44, Del Mar, CA 92014 (US). JOHNSON, David, S.; 9152-L Regents Road, La Jolla, CA 92037 (US). BOULTER, James, Richard; 147 South Rios Avenue, San Diego, CA 92075 (US). HEINEMANN, Stephen, Fox; 8481 Cliffridge Lane, La Jolla, CA 92037 (US).

(74) Agent: REITER, Stephen, E.; Pretty, Schroeder, Brueggemann & Clark, Suite 2000, 444 South Flower Street, Los Angeles, CA 90071 (US).

(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CLONING AND EXPRESSION OF A ACETYLCHOLINE-GATED ION CHANNEL RECEPTOR SUBUNIT

(57) Abstract

The present invention provides isolated nucleic acids encoding alpha9 nicotinic acetylcholine receptor subunit and receptor subunit protein encoded thereby. Also provided are vectors containing the invention nucleic acids, host cells transformed therewith, alpha9 nicotinic acetylcholine receptor subunit and functional nicotinic acetylcholine receptors comprising at least one alpha9 subunit expressed recombinantly in such host cells as well as transgenic non-human mammals that express the invention receptor subunit and mutants thereof. Receptors of the invention comprise at least one alpha9 nicotinic acetylcholine subunit and form cationic channels activated by acetylcholine, but blocked by nicotine and muscarine. The invention also provides methods for identifying compounds that modulate the ion channel activity of the functional invention receptors containing at least one invention subunit.

PCT WORLD INTELLECTUAL Internat

WO 9603504A1

INTERNATIONAL APPLICATION PUBLISHED U

(51) International Patent Classification 6 :
C12N 15/12, 15/85, C07K 14/705, C12N 5/10, C12Q 1/68, C07K 16/28, A01K 67/027, C12Q 1/00

A1

(11) International Publication Number: WO 96/03504

(43) International Publication Date: 8 February 1996 (08.02.96)

(21) International Application Number: PCT/US95/09104

(22) International Filing Date: 18 July 1995 (18.07.95)

(30) Priority Data:
08/278,635 21 July 1994 (21.07.94) US

(71) Applicant: THE SALK INSTITUTE FOR BIOLOGICAL STUDIES [US/US]; 10010 North Torrey Pines Road, La Jolla, CA 92037 (US).

(72) Inventors: ELGOYHEN, Ana, Belen; 1844 Camino Del Mar #44, Del Mar, CA 92014 (US). JOHNSON, David, S.; 9152-L Regents Road, La Jolla, CA 92037 (US). BOULTER, James, Richard; 147 South Rios Avenue, San Diego, CA 92075 (US). HEINEMANN, Stephen, Fox; 8481 Cliffridge Lane, La Jolla, CA 92037 (US).

(74) Agent: REITER, Stephen, E.; Pretty, Schroeder, Brueggemann & Clark, Suite 2000, 444 South Flower Street, Los Angeles, CA 90071 (US).

(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CLONING AND EXPRESSION OF A ACETYLCHOLINE-GATED ION CHANNEL RECEPTOR SUBUNIT

(57) Abstract

The present invention provides isolated nucleic acids encoding alpha9 nicotinic acetylcholine receptor subunit and receptor subunit protein encoded thereby. Also provided are vectors containing the invention nucleic acids, host cells transformed therewith, alpha9 nicotinic acetylcholine receptor subunit and functional nicotinic acetylcholine receptors comprising at least one alpha9 subunit expressed recombinantly in such host cells as well as transgenic non-human mammals that express the invention receptor subunit and mutants thereof. Receptors of the invention comprise at least one alpha9 nicotinic acetylcholine subunit and form cationic channels activated by acetylcholine, but blocked by nicotine and muscarine. The invention also provides methods for identifying compounds that modulate the ion channel activity of the functional invention receptors containing at least one invention subunit.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgystan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SI	Slovenia
CI	Côte d'Ivoire	KZ	Kazakhstan	SK	Slovakia
CM	Cameroon	LI	Liechtenstein	SN	Senegal
CN	China	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	LV	Latvia	TJ	Tajikistan
DE	Germany	MC	Monaco	TT	Trinidad and Tobago
DK	Denmark	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali	UZ	Uzbekistan
FR	France	MN	Mongolia	VN	Viet Nam
GA	Gabon				

CLONING AND EXPRESSION OF A ACETYLCHOLINE-GATED ION CHANNEL RECEPTOR SUBUNIT

BACKGROUND OF THE INVENTION

Intercellular communication is essential for the function of multicellular systems. Ion channel proteins, as mediators of information transfer in the brain, endocrine system, enteric nervous system and neuromuscular junction, modulate ion fluxes that produce voltage changes across cell membranes and simultaneously act as sensors of physiological signals, for example, changes in ligand concentrations and in transmembrane voltage. Ligand-gated ion channels provide for rapid dialogue between cells of the central nervous system, converting a chemical neurotransmitter signal released from one cell into an electrical signal that propagates along the cell membrane of a target cell. Ligand-gated ion channels are multimeric protein complexes with component subunits encoded by related genes.

At the present time, numerous families of ligand-gated receptors have been identified and

characterized on the basis of sequence identity. Those which form cationic channels include, for example, excitatory nicotinic acetylcholine receptors (nAChRs), excitatory glutamate-activated receptors, the 5-HT_3 serotonin receptor, the ATP receptor and the sarcoplasmic ryanodine receptor. Those which form anionic channels include, for example, the inhibitory GABA and glycine-activated receptors.

The neurotransmitter acetylcholine (ACh) activates two pharmacologically different receptor types: nicotinic acetylcholine receptors (nAChR) from the ligand-gated ion channel superfamily and muscarinic acetylcholine receptors (mAChR) from the G-protein coupled receptor superfamily (Taylor, A. Goodman-Gilman, T.H. Rall, A.S. Nies and P. Taylor, eds. (New York:Pergamon Press), pp. 166-186,1990); (Taylor, A. Goodman-Gilman, T.H. Rall, A.S. Nies and P. Taylor, eds. (New York:Pergamon Press), pp. 122-149,1990). A number of pathologies and/or disease conditions are associated with nAChRs, such as, for example, myasthenia gravis, schizophrenia, Alzheimer's disease, Tourette's disease and nicotine addiction. Biochemical and electrophysiological data have shown that nicotinic and muscarinic receptors are functionally distinct entities. (Bonner, et al., *Science*, 237, 527-532, 1987). Whereas nAChRs are pentamers composed of related protein subunits that span the plasma membrane four times, mAChRs are formed by a single polypeptide chain which is postulated to span the plasma membrane seven times.

Nicotinic acetylcholine receptors, glycoproteins composed of five subunits, transduce the binding of acetylcholine in the cationic channel. The five receptor subunits form a pseudosymmetric ring around a central

channel. Neuronal nicotinic AChRs (NnAChRs) mediate neurotransmission at many central and peripheral synapses, and comprise two subunit types (alpha and beta) encoded by 10 different neuronal genes. Expression of particular combinations of subunit RNAs in oocytes yields biophysically distinct channels that are distinguished pharmacologically on the basis of ligands that modulate such channels.

Recombinant DNA technology has enabled the identification of the vertebrate muscle nAChR subunits alpha1, beta1, gamma, delta and epsilon and the neuronal subunits alpha2, alpha3, alpha4, alpha5, alpha6, alpha7, alpha8, beta2, beta3 and beta4 (rat nomenclature). Various combinations of these subunits produce functional recombinant receptor-channel complexes that are activated by both ACh and nicotine. The nAChR at the neuromuscular junction is thought to have a $(\alpha 1)_2\beta 1\gamma\delta$ stoichiometry (Galzi, et al., *Annu. Rev. Pharmacol.*, 31, 37-72, 1991). In contrast, the neuronal nAChR subunits alpha2, alpha3 and alpha4 lead to the assembly of functional nAChRs in concert with either beta2 or beta4 (Boulter, et al. *Proc. Natl. Acad. Sci. USA*, 84, 7763-7767, 1987; Ballivet, et al., *Neuron*, 1, 847-852, 1988; Wada, et al., *Science*, 240, 330-334, 1988; Deneris, et al., *Neuron*, 1, 45-54, 1988; Duvoisin, et al., *Neuron*, 3, 487-496, 1989; Couturier, et al., *J. Biol. Chem*, 265, 17560-17567, 1990), while the neuronal alpha7 and alpha8 subunits can form functional nAChRs in the absence of any other subunit (Couturier, et al., *J. Biol. Chem*, 265, 17560-17567, 1990; Seguela, et al., *J. Neurosci*, 13, 596-604, 1993; Gerzanich, et al., *Molec. Pharmacol.*, 45, 212-220, 1994).

Given the existence of ten distinct nicotinic acetylcholine subunit genes, numerous combinations of subunits producing functional receptors are possible. In spite of the numerous combinations of subunits which can be

prepared from previously cloned genes, the properties of the native nAChRs do not always match those of recombinant receptors (Sargent, *Annu. Rev. Neurosci.*, 16, 403-443, 1993). For example, the cholinergic receptors present in bovine chromaffin cells and in rat and chick cochlear hair cells exhibit a pharmacological profile that does not fit any combination of known subunits (Shirvan, et al., *Proc. Natl. Acad. Sci. USA.*, 88, 4860-4864, 1991; Housley, et al., *Proc. R. Soc. Lond. B,* 244, 161-167, 1991; Fuchs, et al., *Proc. R. Soc. Lond. B,* 248, 35-40, 1992; Erostegui, et al., *Hearing Res.*, 74, 135-147, 1994), thus suggesting the existence of additional, as yet unidentified subunits.

Thus, a need exists for identifying additional members of the nicotinic acetylcholine receptor superfamily, and characterizing such nAChR subunits, as well as functional receptors assembled therefrom, which includes elucidation of the nature of assembly of various subunits in the production of a functional receptor (i.e., a subunit assembly containing ligand binding sites and a ligand-gated transmembrane channel), and the relationship between the structure of the subunit assembly and the pharmacological profile of the corresponding receptor. The present invention satisfies these needs and provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention provides isolated nucleic acids encoding alpha9 nicotinic acetylcholine receptor (nAChR) subunit, isolated receptor subunit protein encoded thereby as well as recombinately expressed alpha9 nicotinic acetylcholine receptor (nAChR). Further provided are vectors and probes containing such nucleic acids, host cells transformed with such nucleic acids, antisense oligonucleotides and compositions containing such oligonucleotides, antibodies that specifically bind to

invention receptors and compositions containing such antibodies as well as transgenic non-human mammals.

The alpha9 nAChR subunits of the invention form a cationic receptor channel complex which is activated by acetylcholine and is permeable to cations, including calcium. Functional alpha9 nACh receptors of the invention may be expressed as homomeric receptors, i.e., only one type of subunit is required for function, or invention receptors may be expressed as heteromeric receptors wherein more than one type of subunit is required to form a functional receptor. Additionally, the invention provides methods for identifying compounds that modulate activity of the invention receptors, or the activity of nucleic acid encoding such receptors.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the nucleotide and the amino acid sequence of the cDNA clone coding for the alpha9 nAChR subunit. The amino acid sequence is shown below the nucleotide sequence. Cleavage of the signal peptide is predicted at amino acid position 1 (Von-Heijne, *Nucl. Acid. Res.*, 14, 4683-4691, 1986). Amino acids encoding the signal peptide are assigned negative numbers. Nucleotides are numbered in the 5' to 3' direction, starting with the first nucleotide of the codon for the putative N-terminal residue of the mature protein. Nucleotides on the 5' side of amino acid residue 1 are indicated by negative numbers. Arrowheads indicate the intron location determined by genomic sequencing. Membrane spanning regions are underlined. The sequence information presented in Figure 1 is also presented in Patentin format in SEQ ID NOs 1 and 2.

Figure 2A shows the restriction map for the alpha9 subunit gene, and Figure 2B shows a partial

restriction map for overlapping genomic clones, M6 and MNANO, spanning the entire coding sequence of the alpha9 subunit gene. *Nco*I and *Nhe*I restriction sites not mapped in pMNANO, and *Sac*I restriction sites are not mapped in pM6.

Figure 3 shows the alignment of amino acid sequences for known nAChR alpha subunits. All sequences correspond to rat subunits, except for alpha8 which is a chick subunit. Identical residues in all sequences are presented as white letters in a black background. Spaces are introduced to maximize homologies. Predicted signal peptides and the four potential membrane spanning regions are indicated. Asterisks denote cysteine residues 127, 141, 191 and 192 (alpha9 numbering for the mature peptide, absent the 28 amino acid residues comprising the signal peptide) conserved in all nAChR alpha subunits.

Figures 4A and 4B show electrophysiological responses of alpha9 injected oocytes to cholinergic agonists. Figure 4A shows current responses elicited by ACh, nicotine, muscarine, 1,1-dimethyl-4-phenylpiperazinium (DMPP), and oxotremorine-M (OXO-M) in oocytes injected with alpha9 cRNA and held at -50 mV under voltage-clamp.

Figure 4B shows concentration-response curves to ACh, DMPP and OXO-M. Values represented are the mean and standard error of the mean of peak current values obtained in at least four oocytes per drug. Error bars are not shown when the standard error is smaller than the symbol. Responses from each cell were normalized to the maximal current evoked by ACh. The Hill equation (EC_{50} = 9.7 μM; slope = 1.3) was fitted to the ACh concentration-responsive curve.

Figures 5A and 5B show the blockage of ACh responses in alpha9 injected oocytes by various

antagonists. Inhibition curves were performed by the coapplication of 10 μM ACh and increasing concentrations of either (-) nicotine or (+) muscarine (see Figure 5A) and strychnine, d-tubocurarine (d-TC) or atropine (see Figure 5B). Responses are expressed as the percentage (%) of the control current evoked by 10 μM ACh. The mean and standard error of the mean of values obtained in at least four different oocytes per drug are shown. Error bars are not shown when the standard error is smaller than the symbol.

Figure 6 shows the sensitivity of ACh-evoked currents in alpha9 injected oocytes to α- and κ-bungarotoxin. Representative current responses to 100 μM ACh recorded at a holding potential of -50 mV are shown. Oocytes were preincubated with α-bungarotoxin (α-BTX, A) or κ-bungarotoxin (κ-BTX, B) for 30 minutes before the application of the second test concentration of ACh.

Figures 7A through 7C show the voltage-dependence of the ACh-evoked currents in alpha9 injected oocytes and Ca^{2+} permeability of the recombinant alpha9 receptor. In Figure 7A the current-voltage relationship of ACh-evoked currents in alpha9-injected oocytes was determined by applying a voltage ramp (2 second duration, +50 mV to -120 mV) during the plateau phase of the current response. The traces are representative of those obtained in four different oocytes.

Figure 7B shows representative current traces elicited by 100 μM ACh in alpha9-expressing oocytes before and after the injection of 50nl of 20mM 1,2-bis (2-aminophenoxy)ethane-N,N,N^1,N^1-tetraacetic acid (BAPTA).

Figure 7C shows ACh-evoked currents in alpha9-injected oocytes held at -10 mV under voltage-clamp and bathed with a Ringer's solution containing 350mM NaCl.

Figure 8 shows detection of alpha9 transcripts in the rat cochlea. Amplification reactions were performed as described in Example VI, using cDNA transcribed from total RNA as template and alpha9 specific primers. Resolution of the amplified products in a 1.5% agarose gel stained with ethidium bromide is shown. An aliquot of 10 μM of each reaction mixture was loaded per lane. Lane 1, DNA ladder; lane 2, no DNA template; lane 3, amplified product from olfactory epithelium cDNA; lane 4, amplified product from olfactory epithelium cDNA; lane 5, amplified product from sciatic nerve cDNA.

Figures 9A through 9F show the results of *in situ* hybridization of sagittal sections of rat embryos and coronal sections of adult brains and identification and transcript localization of alpha9. Figures 9A and 9B show the presence of alpha9 transcripts in the hypophyseal gland, the olfactory epithelium, the sternohyoid muscle and the tongue, of a rat embryo at stage E16. Figures 9C and 9D show a high magnification view of the pituitary in a rat embryo at stage E16 where alpha9 transcript is located in the pars tuberalis but not the pars distalis or the pars nervosa. Figures 9E and 9F show the presence of alpha9 transcripts in the pars tuberalis of the adult rat brain.

DETAILED DESCRIPTION OF THE INVENTION

Molecular cloning studies have demonstrated structural and functional diversity in nicotinic acetylcholine receptors (nAChRs). To date, seven alpha subunits (alpha2 to alpha8) and three beta subunits (beta2 to beta4) have been characterized in the nervous system of vertebrates. The present invention describes the identification and functional characterization of a new member of this family of receptor subunit genes that are activated by the neurotransmitter acetylcholine (ACh). The new member is designated alpha9. The molecular structure

of alpha9 indicates that it belongs to the ionotropic (nicotinic) rather than to the metabotropic (muscarinic) ACh receptor family. However, the mixed nicotinic-muscarinic properties of the recombinant alpha9 receptor differ from the pharmacological profile of all known functional nicotinic receptors.

Isolation and identification of the novel nAChR subunit gene of the present invention was accomplished by screening a rat genomic library using a rat nAChR alpha7 subunit cDNA as probe. DNA sequence analysis revealed that one isolated genomic clone encoded a protein with significant amino acid sequence identity with members of the ligand-gated ion channel gene superfamily. Its homology to known subunits revealed that it was more related to nAChR subunits than to $GABA_A$, glycine or $5\text{-}HT_3$ receptor subunits. The presence of conserved contiguous cysteine residues in the extracellular domain, which are a hallmark of all nAChR alpha subunits and are thought to be part of the acetylcholine binding domain (Popot and Changeux, *Physiol. Rev.* 64, 1162-1193, 1984) suggested that this gene encoded a nAChR alpha subunit. Therefore, in accordance with current nomenclature, this newly discovered subunit has been designated the alpha9 subunit of the nAChR gene family.

A polymerase chain reaction (PCR) fragment derived from the isolated genomic clone was used to screen a rat olfactory epithelium cDNA library. Four independent cDNA clones were isolated, one of which contained a 1937 bp insert encoding an open reading frame for the alpha9 subunit. The nucleotide and deduced amino acid sequences are shown in Figure 1 (and are also presented in Patentin format in SEQ ID NOs 1 and 2). The full length alpha9 cDNA encodes a mature protein of 451 amino acid residues, preceded by a leader sequence of 28 residues. It contains all the features characteristic of other members of the

nAChR gene family, including four hydrophobic regions which predict potential membrane spanning regions, MSR I to IV (Kyte and Doolitle, *J. Mol. Biol.*, 157, 105-132, 1982), and cysteine residues at positions 127, 141, 191 and 192 (alpha9 numbering for the mature peptide, absent the 28 amino acid residues comprising the signal peptide) which are present in all nAChR alpha subunits.

The full-length alpha9 cDNA was used as a probe to screen two mouse genomic libraries constructed in phage vectors lambdaDASH II and lambdaFIX II. Two overlapping genomic clones were obtained (see Figure 2). These clones, spanning the entire coding sequence of the alpha9 subunit gene, were cloned into plasmid vectors and the alpha9 subunit gene structure was determined by sequencing across the intron-exon boundaries. The intron-exon boundaries of the alpha9 gene are indicated in Figure 1. The gene consists of five exons and has an intron-exon structure that differs from that of all known nAChR genes (Noda, et al., *Nature*, 305, 818-823, 1983; Nef, et al., *EMBO J.*, 7, 595-601, 1988; Wada, et al., *Science*, 240, 330-334, 1988; Buonanno, et al., *J. Biol. Chem.*, 264, 7611-7616, 1989; Boulter, et al., *J. Biol. Chem*, 265, 4472-4482, 1990). For example, in contrast to other nAChR subunit genes where the intron-exon boundaries of the first four exons are conserved, exons III and IV of the alpha9 gene are fused.

The alpha9 cDNA clone was sequenced and the sequence compared with sequences of other nAChR alpha subunits (see Figure 3). Based on sequence similarity, the alpha9 subunit appears to be a distant member of the nAChR subunit gene family. It is as distinct from the neuronal alpha7-alpha8 subfamily (38% amino acid sequence identity) as it is from the neuronal alpha2-alpha6 (36-39%) subfamily or from the muscle alpha1 subunit (37%). Although alpha9 shares the most highly conserved sequence elements with other members of the family, some amino acid residues

differ from those found invariant in the other alpha subunits. For example, the conserved hydrophobic residues Phe-99 and Val-230 (alpha9 numbering for the mature peptide, absent the 28 amino acid residues comprising the signal peptide) are changed to the polar residues Ser-99 and Ser-230 in the alpha9 protein and the conserved positively charged residue Lys-144 is substituted by the non-charged residue Thr-144. The hydrophobic residues Leu-255 (alpha1-alpha6 subunits) or Met-255 (alpha7-alpha8 subunits) present in MSR II, are replaced by the polar amino acid Gln-255 in the alpha9 subunit. In addition, when compared to other nAChR subunits, alpha9 has a deletion of a Thr residue between MSR II and MSR III.

A full-length alpha9 cDNA suitable for *Xenopus* oocyte expression studies was constructed by subcloning the fragment from nucleotide -94 to 1766 (Figure 1; i.e., residues 79 to 1938 as presented in SEQ ID NO:1) into the expression vector pGEMHE (Liman et al., *Neuron*, 9, 861-871, 1992). cRNA was synthesized using the mMessage mMachine transcription kit (Ambion, Austin, TX), with plasmid linearized with *Nhe*I.

Two days after the injection of alpha9 cRNA, more than 95% of voltage-clamped *Xenopus* oocytes responded to acetylcholine. Inward currents in response to 100 μM acetylcholine ranged from 20 to 500 nA. Figure 4A shows representative current traces in response to the application of acetylcholine. High concentrations (>10 μM) of this agonist evoked a fast peak response which rapidly decayed to a plateau level. Oocytes expressing alpha9 were insensitive to glutamate, GABA, glycine, serotonin, ATP, histamine and adenosine.

All functional nAChR alpha subunits cloned prior to the cloning of the alpha9 subunit, upon expression in *Xenopus* oocytes, form either heteromeric or homomeric

receptor-channel complexes activated by nicotine (Boulter et al., *Proc. Natl. Acad. Sci. USA*, 84, 7763-7767, 1987; Duvoisin et al., *Neuron*, 3, 487-496, 1989; Couturier et al., *Neuron*, 5, 847-856, 1990; Luetje and Patrick, *J. Neurosci.* 11, 837-845, 1991; Seguela et al., *J. Neurosci.*, 13, 596-604, 1993; Gerzanich et al., *Molec. Pharmacol.*, 45, 212-220, 1994). Strikingly, nicotine (0.1 μM to 1 mM) did not elicit any response in alpha9-injected oocytes (Figure 4A). Co-expression of alpha9 with either beta2 or beta4 nAChR subunits did not result in the formation of receptor-channels that were activated by nicotine. The alpha9 receptor-channel complex was also not activated by muscarine (Figure 4A). Moreover, neither the nicotinic agonist cytosine nor the muscarinic agonists bethanecol and pilocarpine elicited current responses. However, both the nicotinic agonist 1,1-dimethyl-4-phenylpiperazinium (DMPP) and the muscarinic M1 agonist oxotremorine M (OXO-M), induced inward currents in alpha9-injected oocytes (Figure 4A). Figure 4B shows the concentration-response curves to these cholinergic agonists. Acetylcholine had an apparent affinity (EC_{50}) of 10 μM. The maximum current responses elicited by both DMPP and OXO-M were approximately 5% of that observed with acetylcholine.

Although neither nicotine nor muscarine evoked responses in oocytes injected with alpha9 cRNA (see Figure 4A), both of these classic cholinergic agonists reduced the currents evoked by acetylcholine. Figure 5A shows the inhibition curves that resulted from the co-application of 10 μM acetylcholine with increasing concentrations of either nicotine or muscarine (IC_{50} = 30 μM and 75 μM, respectively). As shown in Figure 5B, the alpha9 receptor-channel complex was also blocked by the nicotinic antagonist d-tubocurarine (IC_{50} = 0.3 μM), as well as by the muscarinic antagonist atropine (IC_{50}= 1.3 μM). The alkaloid strychnine, classically used as a blocker of glycine-gated chloride channels, was found to be a potent antagonist of

alpha9 homomers, with an IC_{50} of 0.02 μM (Figure 5B). Both α-bungarotoxin (100 nM) and κ-bungarotoxin (100 nM) blocked responses to 100 μM acetylcholine (Figure 6). The blockade by these toxins was almost completely reversed after a 10 minute wash of the oocytes with frog Ringer's solution.

Electrophysiological properties were determined on alpha9 injected oocytes 2-7 days after injection. The current-voltage (I-V) relationship obtained by the application of a 2 second voltage ramp at the plateau response to acetylcholine is shown in Figure 7A. The I-V curve was non-linear, displaying a maximal inward current elicited by acetylcholine at -50 mV. Current responses were reduced at potentials negative to -50 mV. The fact that the ratio between the inward current elicited by 100 μM acetylcholine and that evoked by 1 μM acetylcholine was greater at -50 mV (2.1) than at -80 mV (1.0), indicates that the reduction in current responses at hyperpolarized potentials may depend upon agonist concentration. At holding potentials more positive than -50 mV, the inward currents activated by acetylcholine decreased until -25 mV, where a strong rectification was observed up to a holding potential of +20 mV. I-V curves for both peak and plateau responses performed with stepwise increments in the holding potential, had the same shape as shown in Figure 7A.

From the I-V relationships, an apparent reversal potential of -25 mV is estimated. This value is compatible with either a non-selective cationic current or with an anionic (Cl^-) current. The change in external NaCl concentration from 50 mM to 150 mM produced a positive shift in the reversal potential of acetylcholine-induced currents. This indicates that the alpha9 channel is permeable to Na^+. Most of the peak response elicited by 100 mM acetylcholine in alpha9-expressing oocytes disappeared when oocytes were injected with the calcium chelator 1,2-bis (2-aminophenoxy)ethane-N,N,N^1,N^1-tetraacetic acid

(BAPTA) (see Figure 7B). Thus, as has been suggested for other nAChR subunits (Gerzanich et al., *Molec. Pharmacol.*, 45, 212-220, 1994), this result indicates that part of the current evoked by acetylcholine is carried by a Cl^- current through Ca^{2+}-activated Cl^- channels known to be present in oocytes (Miledi and Parker, *J. Physiol. (Lond).*, 357, 173-183, 1984). In order to further test the participation of a Ca^{2+} activated Cl^- current in response to acetylcholine, the reversal potentials of Cl^- and Na^+ were shifted in opposite directions by transiently raising the external NaCl concentration to 350 mM and holding the oocytes at -10 mV under two electrode voltage clamp. Under this condition, 100 μM acetylcholine elicited both an outward current followed by an inward current (Figure 7C). As reported for other neuronal nAChRs (Vernino et al., *Neuron*, 8, 127-134, 1992; Seguela et al., *J. Neurosci.*, 12, 596-604, 1993), the inward current probably results from the influx of cations through alpha9 receptor-channels and the outward current from the flux of Cl^- through Ca^{2+}-activated Cl^- channels. It should be noted that I-V curves performed in 1,2-bis(2-aminophenoxy)ethane N,N,N^1,N^1-tetraacetic acid injected oocytes had the same shape as that described above, suggesting that the Cl^- current did not contribute to the I/V curve under the conditions of the experiment.

The above-described *Xenopus* oocyte expression studies demonstrate that the alpha9 protein subunit forms ion channels activated by acetylcholine and permeable to both Na^+ and Ca^{2+}. Similar to the alpha7 and alpha8 neuronal subunits (Couturier et al., *Neuron*, 5, 847-856, 1990; Gerzanich et al., *Molec. Pharmacol.*, 45, 212-220, 1994), alpha9 can assemble into a homomeric receptor-channel complex. This differs from other functional neuronal nAChR alpha subunits which require co-assembly with a beta subunit in order to form receptor-channel complexes (Boulter et al., *Proc. Natl. Acad. Sci. USA*, 84, 7763-7767,

1987; Ballivet et al., *Neuron*, 1, 847-852, 1988; Wada et al., *Science*, 240, 330-334, 1988).

Currents elicited by acetylcholine in alpha9-injected oocytes decreased at holding potentials negative to -50 mV. This could result from a voltage-dependent blockade of the channel either by acetylcholine or by cations present in the solution used to maintain the oocytes. The fact that the block was more pronounced at high agonist concentrations indicates that at least part of this effect is due to a voltage-dependent channel block by acetylcholine. High concentrations of acetylcholine and carbamylcholine are known to produce a voltage- and concentration-dependent channel block of muscle nAChR present in BC_3H-1 cells (Sine and Steinbach, *Biophys. J.*, 46, 277-284, 1984).

Based on its primary structure and electrophysiological properties, the alpha9 protein belongs to the nicotinic family of ligand gated ion channels which includes subunits for nAChR, $GABA_A$, glycine and $5\text{-}HT_3$ receptors. However, as described earlier, in alpha9-injected oocytes, nicotine, muscarine, d-tubocurarine and atropine blocked acetylcholine-evoked current responses. Therefore, the alpha9 receptor-channel complex falls into neither the nicotinic nor the muscarinic subdivisions of the pharmacological classification scheme of cholinergic receptors (P. Taylor in *The pharmacalogical basis of therapeutics*, A. Goodman-Gilman, T.H. Rall, A.S. Nies and P. Taylor, eds. (New York:Pergamon Press), pp. 122-149 and 166-186, 1990). The finding that both the nicotinic agonist DMPP and the muscarinic agonist OXO-M are capable of eliciting current responses in alpha9-injected oocytes indicates that the alpha9 receptor exhibits a mixed nicotinic-muscarinic pharmacology. In addition, the blockage of alpha9 receptors by the glycine receptor antagonist strychnine is unusual. A similar effect of

strychnine has also been reported on alpha7 and alpha8 homomers expressed in *Xenopus* oocytes (Seguela et al., *J. Neurosci.*, 12, 596-604, 1993; Gerzanich et al., *Molec. Pharmacol.*, 45, 212-220, 1994).

The alpha9 protein subunit contains the most conserved amino acid residues within the proposed acetylcholine binding site of nAChR alpha subunits (Dennis et al., *Biochem*, 27, 2346-2357, 1988; Galzi et al., *J. Biol. Chem.*, 265, 10430-10437, 1990). Nevertheless, two non-conservative substitutions in the alpha9 protein, Phe-99 to Ser and Lys-144 to Thr (position numbers refer to the mature protein, absent the 28 residues of the leader sequence), are near the first and second domains of the postulated agonist binding site for nAChR. These amino acid substitutions are likely to be responsible for the distinct pharmacological properties of the alpha9 receptor-channel complex.

To determine the tissue expression pattern of the alpha9 gene, *in situ* hybridization studies were performed. *In vitro* synthesized RNA derived from coding sequence of the alpha9 genomic clone was hybridized to sagittal sections of rat embryos and coronal sections of adult rat brains. The presence of transcripts are observed in the hypophyseal gland of a rat embryo at stage E16 (see Figures 9B and 9D). The alpha9 gene expression is observed to be restricted to the pars tuberalis of the adenohypophysis, whereas the pars distalis and the neurohypophysis show no detectable signal. The alpha9 mRNA is also observed to be present in the adult rat pars tuberalis, at the ventral surface of the median eminence (see Figure 9F). Alpha9 expression is also observed throughout the E16 rat olfactory mucosa (see Figure 9B). The alpha9 transcripts are detected in the pseudostratified columnar epithelium that lines each of the turbinates of the olfactory organ. Additional expression is seen in the tongue of the

developing rat (Figure 9B). Finally, *in situ* hybridization analysis performed on 20 mm coronal sections every 180 mm throughout the adult brain, did not establish alpha9 gene expression in the central nervous system of the rat.

In situ hybridization studies performed on cryostat sections of the rat cochlea indicate that the alpha9 gene is also expressed in the outer hair cell region of all cochlea turns. No expression of the alpha9 gene was observed in spiral ganglion neurons or other supporting structures of the cochlea (see Figure 9B).

Previously published neuronal nAChR genes are reported as being expressed in the central nervous system of vertebrates (Sargent, *Annu. Rev. Neurosci.*, 16, 403-443, 1993). As disclosed above, *in situ* hybridization studies performed in coronal sections throughout the rat brain did not establish alpha9 gene expression in the central nervous system. Although low levels of alpha9 transcripts or a very restricted expression pattern which escaped detection cannot be ruled out, the results suggest that relative to other nAChR subunits, alpha9 may be involved in a distinct subset of cholinergic functions *in vivo*. *In situ* hybridization studies showed that in the rat the alpha9 subunit gene is expressed in the pars tuberalis of the pituitary, the olfactory epithelium, the outer hair cells of the cochlea and the skeletal muscle of the tongue.

The pars tuberalis constitutes an anatomically well defined part of the vertebrate adenohypophysis consisting of peptide-secreting cells, gonadotropes and thyrotropes (Wittkowski et al., *Acta Endocrinol.*, 126, 285-290, 1992). Neuroendocrine effects, such as the inhibition of luteinizing hormone and thyroid-stimulating hormone secretion in response to nicotine exposure, has been reported in humans and rats (Fuxe et al., *Psychoneuroendocrinol.*, 14, 19-41, 1989). Although these

effects have been attributed to the activation of hypothalamic nAChR, the presence of the alpha9 nAChR subunit in the pituitary indicates that nicotine may exert a direct action on this gland.

It is likely that the olfactory sensory cells receive efferent innervation that modulates olfactory function (Shirley, *Olfaction. Intl. Rev. Neurobiol.*, 33, 1-53, 1992). A cholinergic modulation has been suggested, since the application of acetylcholine causes slow electrical potentials and modifies spike activity in olfactory receptor neurons (Bouvet et al., *Neurosci. Res.*, 5, 214-223, 1988). Although further pharmacological characterization of the acetylcholine response in olfactory neurons as well as a more precise localization of the alpha9 subunit within the olfactory epithelium are necessary, the presence of alpha9 transcripts in the olfactory epithelium could provide the molecular basis for the cholinergic effect described.

The alpha9 gene expression in the developing muscle of the tongue is intriguing. With the *in situ* hybridization studies performed it is not possible to distinguish whether the signal is actually localized in muscle fibers or in the surrounding connective tissue. However, alpha9 transcripts appear not to be present in all developing skeletal muscles. For example, *in situ* hybridization studies performed in mid-sagittal sections of rat embryos showed no evidence of alpha9 transcripts in the intercostal or axial muscles.

The overall pharmacological characteristics of the homomeric alpha9 receptor expressed in oocytes differ from those of other cloned nAChRs (Boulter, et al., *Proc. Natl. Acad. Sci. USA*, 84, 7763-7767, 1987; Ballivet, et al., *Neuron*, 1, 847-852, 1988; Wada, et al., *Science*, 240,

330-334, 1988; Couturier, et al., *Neuron*, 5, 847-856, 1990; Gerzanich, et al., *Molec. Pharmacol.*, 45, 212-220, 1994).

In order to further investigate the expression pattern of the alpha9 gene in the rat cochlea, PCR was performed on cDNA reverse transcribed from cochlear total RNA. Two primers specific for the alpha9 sequence were designed and used to amplify a fragment that spans an intron-exon boundary of the alpha9 gene. As shown on Figure 8, a fragment of the expected size (573 bp) was amplified from rat cochlear cDNA with alpha9 primers. Restriction endonuclease analysis of the fragment with *Acc*I, *Hinf*I and *Nco*I, further confirmed that it was derived from alpha9 transcripts. Since the alpha9 gene is also transcribed in the rat olfactory epithelium, RNA obtained from this tissue was used as a positive control. Rat sciatic nerve cDNA was included as a negative control to rule out the possibility that, with the parameters used for the PCR, very low levels of transcripts would be detected in any tissue studied. Whereas no DNA was amplified from the sciatic nerve using specific primers for alpha9 (see Figure 8), both alpha3 and alpha4 subunits could be detected in this tissue with their respective specific primers.

A possible physiological role for the alpha9 receptor-channel is the efferent cholinergic innervation of cochlea hair cells. Outer hair cells of the cochlea are implicated in the mechanical amplification of sound in vertebrates (Flock, R. Klinke and R. Hartmann, eds. (Berlin:Springer-Verlag), pp. 2-8, 1983). These cells receive efferent cholinergic innervation. The electrical stimulation of these efferent neurons results in reduction of sensitivity and tuning of the auditory nerve fibers, which in turn could induce protection against acoustic trauma (Brown and Nuttal, *J. Physiol. (Lond.)*, 354, 625-646, 1984; Klinke, *Hearing Res.*, 22, 235-243, 1986;

Rajan and Johnstone, *Brain Res.*, 458, 241-255, 1988). The molecular nature of the acetylcholine receptor involved in the efferent innervation of cochlear hair cells has not been described. Although both a non-selective cation channel as well as a G-protein coupled receptor have been proposed, cholinergic agonists and antagonists have been of little benefit to characterize this receptor as either nicotinic or muscarinic (Housley and Ashmore, *Proc. R. Soc. Lond. B*, 244, 161-167, 1991; Fuchs and Murrow, *Proc. R. Soc. Lond. B*, 248, 35-40, 1992; Fuchs and Murrow, *J. Neurosci.*, 12, 800-809, 1992; Kakehata et al., *J. Physiol. (Lond.)*, 463-, 227-244, 1993; Erostegui et al., *Hearing Res.*, 74, 135-147, 1994). Therefore, whatever the primary structure for this cholinergic receptor might be, based on its unique pharmacological characteristics it has been suggested that it is of a receptor type not previously described (Fuchs and Murrow, *Proc. R. Soc. Lond. B*, 248, 35-40, 1992; Erostegui et al., *Hearing Res.*, 74, 135-147, 1994).

The results presented herein suggest that the alpha9 receptor is the cholinergic component of the cochlear efferent system. This conclusion is based primarily on the presence of alpha9 transcripts in the hair cells of the rat cochlea. Evidence to date suggests that the cochlear efferent system is involved in improving the detection of signal within background noise, protection of the cochlea from noise damage, and attenuating the cochlear response to auditory stimulation when attention must be focused elsewhere.

Various experiments have shown that the cholinergic component of the cochlear efferent system may also be involved in aminoglycoside antibiotic ototoxicity. When administered in high doses, these antibiotics cause outer hair cells (OHC) to degenerate (Govaerts, et al., *Toxicology Letters*, 52, 227-251, 1990). The results of

such degeneration ranges from ringing in the ears to total loss of hearing. Current theories regarding the mechanisms whereby aminoglycosides exert their ototoxic effect upon the OHCs suggest that the OHCs become metabolically destabilized due to a block of intracellular messaging systems. At the same time, the efferent synapses are also destabilized, and can no longer monitor and control the amount of ACh released following stimulation. The end result is that there is an overstimulation (an excess of ACh) directed toward the destablized OHCs, which results in the degeneration observed (Williams, et al., *Hearing Res.*, 30, 11-18, 1987). Thus, ACh, and the alpha9 receptor responsible for transducing the efferent signal from the efferent terminal to the hair cell, are intimately involved in releasing the ototoxic potential of the aminoglycoside antibiotics. Accordingly, antagonists to receptors comprising at least one alpha9 receptor subunit (i.e., alpha9 blockers) will reduce or eliminate the side-effects of aminoglycoside-induced ototoxicity.

The present invention provides isolated nucleic acids encoding an alpha9 nicotinic acetylcholine receptor subunit. The term "nucleic acids" (also referred to as polynucleotides) encompasses RNA as well as single and double-stranded DNA and cDNA. As used herein, the phrase "isolated polynucleotide" refers to a polynucleotide that has been separated or removed from its natural environment. One means of isolating a polynucleotide encoding an alpha9 nAChR receptor subunit is to probe a mammalian genomic library with a DNA probe using methods well known in the art. DNA probes derived from the alpha9 receptor gene particularly useful for this purpose. DNA and cDNA molecules that encode alpha9 receptors can be used to obtain complementary genomic DNA, cDNA or RNA from human, mammalian, or other animal sources. Such molecules can also be used to isolate related cDNA or genomic clones by screening cDNA or genomic libraries, by methods described

in more detail below. Invention nucleic acids include nucleotide sequences that are substantially the same as the nucleotide sequence shown in Figure 1 (see also SEQ ID NO:1). The present invention also encompasses nucleic acids which are degenerate variants of the nucleotide sequence shown in Figure 1 (and SEQ ID NO:1).

The term "degenerate variants" refers to nucleic acids encoding alpha9 nAChR subunits that, by virtue of the degeneracy of the genetic code, do not necessarily hybridize to the invention nucleic acids under specified hybridization conditions. Preferred nucleic acids encoding invention polypeptide(s) or proteins(s) are comprised of nucleotides that encode substantially the same amino acid sequence set forth in Figure 1 (see also SEQ ID NO:2). Alternatively, preferred nucleic acids encoding invention polypeptide(s) hybridize under high stringency conditions to substantially the entire sequence, or substantial portions (i.e., typically at least 25-30 contiguous nucleotides) of the nucleotide sequence set forth in Figure 1 (see also SEQ ID NO:1).

Stringency of hybridization, as used herein, refers to conditions under which polynucleotide hybrids are stable. As known to those of skill in the art, the stability of hybrids is a function of sodium ion concentration and temperature. (See, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual 2d Ed. (Cold Spring Harbor Laboratory, 1989; incorporated herein by reference).

The invention provides isolated alpha9 nicotinic acetylcholine receptor subunit peptide, polypeptide and/or protein encoded by invention nucleic acids and alpha9 nicotinic acetylcholine receptor comprising said subunit. The alpha9 nAChR subunit comprises a protein of approximately 451 amino acids in length. The amino acid

sequence of the alpha9 subunit is set forth in Figure 1 (and in SEQ ID NO:2).

As used herein, the term "isolated protein" refers to a protein free of cellular components and/or contaminants normally associated with a protein in its native *in vivo* environment. Invention polypeptides and/or proteins include naturally occurring allelic variants, as well as recombinant forms thereof. The alpha9 nAChR polypeptide can be isolated using various methods well known to those of skill in the art. The methods available for the isolation and purification of invention proteins include, precipitation, gel filtration, ion-exchange, reverse-phase and affinity chromatography. Other well-known methods are described in Deutscher et al., Guide to Protein Purification: Methods in Enzymology Vol. 182, (Academic Press, 1990), which is incorporated herein by reference. Alternatively, isolated polypeptides of the present invention can be obtained using well-known recombinant methods as described, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual 2d Ed. (Cold Spring Harbor Laboratory, 1989; incorporated herein by reference).

Invention polypeptide(s) can be produced by expressing nucleic acids encoding the alpha9 nAChR subunit in a suitable host cell, such as a bacterial cell, a yeast cell, an amphibian cell (i.e., oocyte), or a mammalian cell, using methods well known in the art. The expressed polypeptide can be recovered using well-known methods. Invention polypeptides can be isolated directly from cells that have been transformed with expression vectors, described below in more detail. The invention polypeptide, biologically active fragments, and functional equivalents thereof can also be produced by chemical synthesis. As used herein, "biologically active fragment" refers to any portion of the alpha9 polypeptide represented by the amino

acid sequence in Figure 1 (see also SEQ ID NO:2) that can assemble into a cationic channel activated by acetylcholine and permeable to calcium. Synthetic polypeptides can be produced, for example, using Applied Biosystems, Inc. Model 430A or 431A automatic peptide synthesizer (Foster City, CA) employing the chemistry provided by the manufacturer.

As used herein, the phrase "nicotinic acetylcholine receptor (nAChR) subunit" refers to recombinantly expressed/produced (i.e., isolated or substantially pure) protein that contains four highly hydrophobic regions which predict membrane spanning regions and cysteine residues at positions 127, 141, 191 and 192 (referring to the mature peptide, not including the 28 amino acid leader sequence). Such protein subunits assemble into a cationic channel which is activated by acetylcholine. Invention nAChR subunits include variants thereof encoded by mRNA generated by alternative splicing of a primary transcript, as well as biologically active fragments.

The alpha9 nAChR subunit of the invention contributes to the formation of a functional receptor, as assessed by methods described herein, by combining with at least one additional nAChR subunit of the same or different type. As used herein, the phrase "functional receptor" means that the binding of a ligand, for example, acetylcholine (ACh), causes the receptor ion channel to open thereby permitting cations, such as Ca^{2+}, as well as Na^{+} and K^{+}, to enter the cell. Agonist activation of a "functional invention receptor" induces the receptor.

Modification of invention nucleic acids, polypeptides or proteins with the following phrases: "recombinantly expressed/produced", "isolated", or "substantially pure", encompasses nucleic acids, peptides, polypeptides or proteins that have been produced in such

form by the hand of man, and are thus separated from their native *in vivo* cellular environment. As a result of this human intervention, the recombinant nucleic acids, polypeptides and proteins of the invention are useful in ways that the corresponding naturally occurring molecules are not, such as identification of compounds as potential drugs.

Sequences having "substantial sequence homology" are intended to refer to nucleotide sequences that share at least about 90% identity with invention nucleic acids; and amino acid sequences that typically share at least about 95% amino acid identity with invention polypeptides. It is recognized, however, that polypeptides or nucleic acids containing less than the above-described levels of homology arising as splice variants or generated by conservative amino acid substitutions, or by substitution of degenerate codons are also encompassed within the scope of the present invention.

The present invention also provides nucleic acids encoding alpha9 receptor subunit(s) operatively linked to a promoter, as well as other regulatory sequences. As used herein, the phrase "operatively linked" refers to the functional relationship of the nucleic acid with regulatory and effector sequences, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. Specifically operative linkage of a nucleic acid to a promoter refers to the physical and functional relationship between the nucleic acid and the promoter such that transcription of DNA is initiated from the promoter by an RNA polymerase that specifically recognizes and binds to the promoter.

Suitable promoters include specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. Additionally, suitable

promoters include sequences that modulate the recognition, binding and transcription initiation activity of RNA polymerase. Such sequences may be *cis* acting or may be responsive to *trans* acting factors. Depending upon the nature of the regulation, promoters may be constitutive or regulated. Examples of promoters are SP6, T4, T7, SV40 early promoter, cytomegalovirus (CMV) promoter, mouse mammary tumor virus (MMTV) steroid-inducible promoter, Moloney murine leukemia virus (MMLV) promoter, and the like.

Vectors employed in the present invention contain both a promoter and a cloning site into which nucleic acid encoding alpha9 receptor subunit(s) can be operatively linked. Such vectors, which are well known in the art, are capable of transcribing RNA *in vitro* or *in vivo*, and are commercially available from sources such as Stratagene (La Jolla, CA) and Promega Biotech (Madison, WI). In order to optimize expression and/or *in vitro* transcription, it may be necessary to remove, add or alter 5' and/or 3' untranslated portions of the clones to eliminate extra, potentially inappropriate alternative translation initiation codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites can be inserted immediately 5' of the start codon to enhance expression. (See, for example, Kozak, *J. Biol. Chem.* 266:19867 (1991)). Similarly, alternative codons, encoding the same amino acid, can be substituted for native codons of the alpha9 nAChR subunit in order to enhance transcription (e.g., the codon preference of the host cell can be adopted, the presence of G-C rich domains can be reduced, and the like).

Examples of suitable vectors that may be employed in the present invention include viruses, such as baculoviruses and retroviruses, bacteriophages, cosmids,

plasmids and other recombination vehicles typically used in the art. Invention nucleic acids are inserted into vector genomes using methods well known in the art. For example, insert and vector DNA can be contacted, under suitable conditions, with a restriction enzyme to create complementary ends on each molecule that can pair with each other and be joined together with a ligase. Alternatively, synthetic linkers can be ligated to the termini of restricted invention nucleic acids. These synthetic linkers contain nucleic acid sequences that correspond to a particular restriction site in the vector DNA. Additionally, a nucleic acid containing a termination codon and an appropriate restriction site can be ligated into a vector containing, for example, some or all of the following: a selectable marker gene, such as the neomycin gene for selection of stable or transient transfectants in mammalian cells; enhancer/promoter sequences from the immediate early gene of human CMV for high levels of transcription; transcription termination and RNA processing signals from SV40 for mRNA stability; SV40 polyoma origins of replication and ColE1 for proper episomal replication; versatile multiple cloning sites; and T7 and SP6 RNA promoters for *in vitro* transcription of sense and antisense RNA. Other means are well known and available in the art.

Also provided are vectors comprising nucleic acid encoding alpha9 nAChR subunit, which vectors are adapted for expression in a bacterial cell, a yeast cell, an amphibian cell (i.e., oocyte), a mammalian cell or other animal cells. Such vectors additionally comprise regulatory elements necessary for expression of nucleic acid in the bacterial, yeast, amphibian, mammalian or animal cells located relative to the nucleic acid encoding alpha9 nAChR subunit so as to permit expression thereof. As used herein, "expression" refers to the process by which nucleic acids are transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the nucleic acid

is derived from genomic DNA, expression may include splicing of the mRNA, if an appropriate eucaryotic host is selected. Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector might include a promoter such as the lac promoter, the Shine-Dalgarno transcription initiation sequence and the start codon AUG (Sambrook et al., Molecular Cloning: A Laboratory Manual 2d Ed. (Cold Spring Harbor Laboratory, 1989; incorporated herein by reference) Similarly, a eucaryotic expression vector might include a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors can be obtained commercially or assembled from available sequences and by methods well known in the art.

This invention also provides a transformed host that expresses recombinant alpha9 nicotinic acetylcholine receptor. Such a host has been transformed with a nucleic acid encoding alpha9 nAChR subunit. An example of a transformed host according to the present invention is a mammalian cell comprising a plasmid specifically adapted for expression in such a cell. The plasmid contains a nucleic acid encoding an alpha9 nAChR subunit and the regulatory elements necessary for expression of the subunit. Suitable mammalian cells that may be utilized in the present invention include, for example, mouse fibroblast NIH3T3 cells, CHO cells, HeLa cells, Ltk$^-$ cells, PC12 and N2A neuronal cells, HEK-293 kidney cells and CG4 glial cells. Host cells may be transformed with plasmids such as those described *supra* by methods well known in the art such as calcium phosphate precipitation, DEAE-dextran, electroporation, microinjection or lipofection. Other suitable hosts that may be employed in the present invention are oocytes, particularly *Xenopus* oocytes.

Nicotinic acetylcholine receptors, according to the invention, are recombinantly expressed in a host cell containing at least one alpha9 subunit. Recombinant receptors may be homomeric or heteromeric. Thus, a transformed host cell can recombinantly express a receptor containing only alpha9 subunits, or containing at least one alpha9 subunit and one or more other nAChR subunits.

The present invention also provides nucleic acid probes. Such probes comprise a polynucleotide capable of specifically hybridizing with a sequence encoding an alpha9 nAChR subunit. As used herein, the term "probe" refers to single-stranded or double-stranded DNA or RNA that has a sequence of nucleotides that includes at least 14 contiguous bases set forth in Figure 1 (see also SEQ ID NO:1). Probes used to distinguish the alpha9 subunit from other alpha nAChRs subunits will preferably consist of at least 14 contiguous bases from the cytoplasmic loop region of the alpha9 nucleotide sequence. Alternatively, probes that are to be used to find additional subunits of the nAChR family will preferably consist of at least 14 contiguous bases from a membrane spanning region of the alpha9 nucleotide sequence.

As used herein, the phrase "specifically hybridizing" encompasses the ability of a polynucleotide to recognize a nucleic acid sequence that is complementary thereto and to form double-helical segments via hydrogen bonding between complementary base pairs. Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary greatly in length and may be labeled with a detectable agent, such as a radioisotope, a fluorescent dye, and the like, to facilitate detection of the probe. Invention probes are useful to detect the presence of nucleic acids encoding the alpha9 nAChR subunit. For example, the probes can be used for *in situ* hybridizations to identify specific

tissues in which the alpha9 nAChR subunit gene is expressed. Additionally, oligonucleotides complementary to nucleic acids encoding the alpha9 nAChR subunit are useful for detecting the alpha9 gene and associated mRNA, or for the isolation of related genes using homology screening of genomic or cDNA libraries, or by using amplification techniques well known to those of skill in the art.

The invention further provides antisense oligonucleotides having a sequence capable of binding specifically with any portion of an mRNA that encodes the alpha9 nAChR subunit so as to prevent translation of the mRNA. Antisense oligonucleotides may also contain a sequence capable of binding specifically with any portion of the cDNA encoding the alpha9 subunit. As used herein, the phrase "binding specifically" encompasses the ability of a nucleic acid sequence to recognize a complementary nucleic acid sequence and to form double-helical segments therewith via the formation of hydrogen bonds between the complementary base pairs.

Also provided by the present invention are compositions comprising an amount of an invention antisense oligonucleotide effective to reduce expression of the alpha9 nAChR subunit wherein said antisense oligonucleotide is capable of binding with mRNA encoding the alpha9 nAChR receptor so as to prevent its translation. Compositions provided by the present invention comprise an acceptable hydrophobic carrier capable of passing through cell membranes and may also comprise a structure which binds to a receptor specific for a selected cell type and is thereby taken up by cells of the selected cell type. The structure may be part of a protein known to bind to a cell-type specific receptor.

Antisense oligonucleotide compositions (AOCs) according to the present invention are designed to be

stable in the blood stream for administration to a subject by injection, or in laboratory cell culture conditions. The physical and chemical properties of the AOC are selected so that the composition is capable of passing through the cell membrane in order to enter the cell cytoplasm. Such a composition can be designed to include small, hydrophobic chemical structures, or alternatively, specific cell transport systems which facilitate and transport the AOC into the cell. In addition, the AOC can be designed for administration only to certain selected cell populations by targeting the AOC to be bound and taken up by select cell populations. Targeting can be accomplished by designing cell specific AOCs to bind to a receptor found only in a certain cell type, as discussed *supra*. Alternatively, an AOC can also be designed to recognize and selectively bind to a target mRNA sequence. In the latter instance, targeting is accomplished, for example, by employing a sequence contained within the sequence shown in Figure 1 (SEQ ID NO:1). The AOC is designed to inactivate the target mRNA sequence by (1) binding to target mRNA and inducing degradation of the mRNA by, for example, RNase I digestion, or (2) inhibiting translation of target mRNA by interfering with the binding of translation-regulating factors or ribosomes, or by inclusion of other chemical structures, such as ribozyme sequences or reactive chemical groups which either degrade or chemically modify the target mRNA. AOCs have been shown to be capable of such properties when directed against mRNA targets (see Cohen et al., *TIPS,* 10:435 (1989) and Weintraub, *Sci. American*, January (1990), pp. 40; both incorporated herein by reference).

The invention also provides antibodies having specific reactivity with alpha9 nAChR polypeptides and/or proteins of the subject invention. Active fragments of antibodies are encompassed within the definition of "antibody".

The antibodies of the invention can be produced by methods known in the art. For example, polyclonal and monoclonal antibodies can be produced by methods described, for example, in Harlow and Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory 1988), which is incorporated herein by reference. The alpha9 protein of the invention, or portions thereof, can be used as the immunogen in generating such antibodies. Alternatively, synthetic peptides can be prepared (using commercially available synthesizers) and used as immunogens. Amino acid sequences can be analyzed by methods well known in the art to determine whether they encode hydrophobic or hydrophilic domains of the corresponding alpha9 invention protein. Altered antibodies such as chimeric, humanized, CDR-grafted or bifunctional antibodies can also be produced by methods well known in the art. Such antibodies can also be produced by hybridoma, chemical synthesis or recombinant methods described, for example, in (Sambrook et al., *Molecular Cloning: A Laboratory Manual* 2d Ed. (Cold Spring Harbor Laboratory, 1989); incorporated herein by reference and Harlow and Lane, *supra*). Both anti-peptide and anti-fusion protein antibodies can be used. (see, for example, Bahouth et al., *Trends Pharmacol. Sci.* 12:338 (1991); Ausubel et al., *Current Protocols in Molecular Biology* (John Wiley and Sons, NY 1989) which are incorporated herein by reference).

The invention antibodies have various uses, such as, for example, isolation of the alpha9 invention receptor. Additionally, the antibodies are useful for detecting the presence of the alpha9 receptor, as well as analysis of receptor localization, subunit composition, and structure of functional domains. A method for detecting the presence of alpha9 nAChRs on the surface of a cell comprises contacting the cell with an antibody that specifically binds alpha9 nACh receptor and detecting the presence of the bound antibody on the cell surface. With

respect to the detection of alpha9 receptors, the invention antibodies can be used, for example, for *in vitro* diagnostic or *in vivo* imaging methods.

Immunological procedures useful for *in vitro* detection of alpha9 receptor in a sample include immunoassays that employ a detectable antibody. Such immunoassays include, for example, ELISA, Pandex microfluorimetric assay, agglutination assays, flow cytometry, serum diagnostic assays and immunohistochemical staining procedures which are well known in the art. An antibody can be made detectable by various means well known in the art. For example, a detectable marker can be directly or indirectly attached to the antibody. Useful markers include, for example, radionuclides, enzymes, fluorogens, chromogens and chemiluminescent labels.

Further, invention antibodies can be used to modulate the ion channel activity of the alpha9 receptor in animals and humans as well as biological tissues isolated therefrom. Accordingly, the invention provides compositions comprising a carrier and an amount of an antibody having specificity for the alpha9 receptor effective to block binding of naturally occurring ligands to the receptor. A monoclonal antibody directed to an epitope of an alpha9 receptor present on the surface of a cell wherein said antibody has an amino acid sequence substantially the same as an amino acid sequence set forth in Sequence ID No. 2 can be useful for this purpose.

The invention further provides a transgenic non-human mammal capable of expressing nucleic acid encoding alpha9 protein. Also provided are transgenic non-human mammals incapable of expressing nucleic acid encoding biologically functional alpha9 protein or alternatively, capable only of expressing alpha9 protein that is biologically deficient in some respect. Varying

degrees of disfunctionality are achieved through manipulation of alpha9 nucleic acid to encode a mutated protein.

The present invention also provides a transgenic non-human mammal having a genome comprising antisense nucleic acid which is transcribed into antisense mRNA complementary to alpha9 mRNA. Such antisense mRNA hybridizes to alpha9 mRNA and reduces the translation thereof.

Nucleic acids employed in transgenic animals of the invention may be associated with an inducible promoter and/or tissue specific regulatory elements, so that expression can be induced, or restricted to specific cell types. Examples of suitable promoters are the metallothionein promoter and the L7 promoter.

The transfer of nucleic acid material into mammalian hosts for the purpose of generating transgenic animals can be accomplished by microinjection, retroviral infection or other means well known to those skilled in the art, of the material into appropriate fertilized embryos. (See, for example, Hogan et al., *Manipulating the Mouse Embryo: A Laboratory Manual* (Cold Spring Harbor Laboratory, 1986). Homologous recombination can also be used for the generation of transgenic animals according to the present invention. Homologous recombination techniques are well known in the art. Homologous recombination replaces a native (endogenous) gene with a recombinant or mutated gene to produce an animal that cannot express a native (endogenous) alpha9 receptor subunit but can express, for example, a mutated receptor subunit. In contrast to homologous recombination, microinjection adds genes to the host genome, without removing host genes. Microinjection can produce a transgenic animal that is capable of expressing both endogenous and exogenous alpha9 receptor

subunits. Transgenic animal model systems are useful for *in vivo* screening of compounds for identification of receptor-specific ligands, i.e., agonists and antagonists, which activate or inhibit receptor responses.

Nucleic acids, oligonucleotides (including antisense), vectors containing same, transformed hosts, receptor subunits and combinations thereof, as well as antibodies of the present invention, can be used to screen compounds *in vitro* to identify those compounds which function as agonists or antagonists of alpha9 receptor subunits of the invention. Such *in vitro* screening assays provide useful information regarding the function and activity of alpha9 receptor subunits of the invention, which can facilitate the identification and design of drugs that are capable of specific interaction with one or more types of receptor subunits or receptor subtypes.

The present invention also provides a method for identifying compounds which bind to alpha9 nicotinic acetylcholine receptor subunits. In such a method, invention receptor subunits may be employed in a competitive binding assay. Such an assay can accommodate the rapid screening of a large number of compounds to determine which compounds, if any, are capable of binding to the alpha9 nAChR subunit. Subsequently, more detailed assays can be carried out with those compounds found to bind, to further determine whether such compounds act as agonists or antagonists of invention receptors (i.e., nAChRs comprising at least one alpha9 subunit).

The present invention still further provides a bioassay for identifying compounds which modulate the activity of receptors of the invention (i.e., nAChRs comprising at least one alpha9 subunit). In one embodiment, the bioassay is conducted by providing cells expressing receptor comprising at least one alpha9 subunit

with at least one potential agonist and thereafter monitoring the cells for changes in ion channel activity. In yet another embodiment, the bioassay is conducted by contacting cells expressing receptor comprising at least one alpha9 subunit with a constant amount of a known alpha9 agonist and increasing amounts of at least one potential antagonist and thereafter monitoring the cells for changes in ion channel activity.

The present invention also provides a bioassay for identifying compounds which modulate the regulatory regions of the alpha9 nAChR subunit gene. Such an assay is conducted utilizing mammalian cells transformed with a nucleic acid construct comprising at least a portion of the regulatory region of the alpha9 gene operatively associated with a reporter gene. The transformed cells are contacted with at least one compound wherein the ability of said compound to modulate the regulatory region is unknown. Thereafter, the cells are monitored for expression of the reporter gene. Suitable reporter genes that may be employed include, for example, the chloramphenicol acetyltransferase gene, the luciferase gene, and the like.

A compound or a signal that "modulates the activity" of an invention receptor refers to a compound or a signal that alters the activity of the alpha9 receptor so that the receptor is different in the presence of the compound or signal than in the absence of the compound or signal. Compounds affecting modulation include agonists and antagonists. An agonist encompasses a compound such as acetylcholine, that activates alpha9 receptor function. Alternatively, an antagonist includes a compound that interferes with alpha9 receptor function. Typically, the effect of an antagonist is observed as a blocking of agonist-induced receptor activation. Antagonists include competitive as well as non-competitive antagonists. A competitive antagonist (or competitive blocker) interacts

with or near the site specific for agonist binding. A non-competitive antagonist or blocker inactivates the function of the receptor by interacting with a site other than the agonist interaction site.

As understood by those of skill in the art, bioassay methods for identifying compounds that modulate nAChR activity generally require comparison to a control. One type of "control" is a cell or culture that is treated substantially the same as the test cell or test culture exposed to the compound, with the distinction that the "control" cell or culture is not exposed to the compound. For example, in methods that use voltage clamp electrophysiological procedures, the same cell can be tested in the presence or absence of compound, by merely changing the external solution bathing the cell. Another type of "control" cell or culture that can be employed is a cell or culture that is identical to transfected cells, with the exception that the "control" cell or culture does not express functional alpha9 nACh receptor subunit. Accordingly, the response of the transfected cell to compound is compared to the response (or lack thereof) of the "control" cell or culture to the same compound under the same reaction conditions.

In still another embodiment of the present invention, the ion channel activity of alpha9 nAChR can be modulated by contacting the receptors with an effective amount of at least one compound identified by any of the above-described bioassays.

The following examples are intended to illustrate, but not limit, the present invention.

EXAMPLE I

Screening of Genomic Libraries

A full-length alpha7 nAChR subunit cDNA (Seguela, et al., *J. Neurosci.*, 13, 596-604, 1993) was used to screen 5 x 10^5 clones of a lambdaCharon 4A rat genomic library (obtained from Dr. James Eberwine, Department of Pharmacology, University of Pennsylvania Medical School, Philadelphia, PA). Hybridization was carried out at 65°C in 1 M NaCl, 50 mM Tris-HCl, pH 8.0, 0.5% SDS, 100 mg/ml denatured salmon sperm DNA and 0.1% (w/v) each of Ficoll, polyvinylpyrrolidone and bovine serum albumin. Filters were washed at 45°C in 2 x SSPE (1 x SSPE is 180mM NaCl, 9mM Na_2HPO_4, 0.9mM NaH_2PO_4 and 1mM EDTA, pH 8.0). A clone of ~16kb containing exons IV and V of the alpha9 subunit gene was isolated.

EXAMPLE II

Screening of a cDNA Library

A PCR fragment derived from coding sequences (nucleotides 283 to 806, Figure 1; i.e., nucleotides 455 to 979 of SEQ ID NO:1) of the rat genomic clone described in EXAMPLE I was used as a probe to screen 1 x 10^6 plaques of a lambdaNM1149 adult rat olfactory epithelium cDNA library (obtained from Dr. Heinz Breer, and Dr. Klaus Raming, University Stuttgart-Hohenheim, Institute of Zoophysiology, Stuttgart, Germany). Hybridization was as described in EXAMPLE I and filters were washed at 65°C in 0.2 x SSPE. Four independent clones were isolated, one contained a full-length alpha9 cDNA (Figure 1). The alpha9 cDNA consists of an 87 bp 5' untranslated region, an open reading frame of 1437 bp and 413 bp of 3' untranslated region. The full length alpha9 cDNA was used as a probe to screen two mouse (129SvJ) genomic libraries constructed in phage vectors lambda DASHII and lambda FIXII. Two overlapping genomic clones were obtained (Figure 2). These

clones, spanning the entire coding sequence of the alpha9 subunit gene were cloned into plasmid vectors and the alpha9 subunit gene structure was determined by sequencing across the intron-exon boundaries.

EXAMPLE III

Nucleotide Sequence Determination and Analysis

The alpha9 subunit cDNA clone was sequenced using the Sequenase 2.0 kit (United States Biochemical, Cleveland, OH) and synthetic oligonucleotide primers. A comparison of the alpha9 amino acid sequences with other nAChR alpha subunits was made using sequence analysis software from the University of Wisconsin Genetics Computer Group [Devereux, et al., *Nucl. Acids. Res.*, 12, 387-395, 1984]. The percent sequence identity between paired sequences was calculated by dividing the number of identical residues by the total number of residues in the shorter of the sequences and multiplying the quotient by 100.

EXAMPLE IV

Electrophysiological Procedures

A full-length alpha9 cDNA suitable for *Xenopus* oocyte expression studies was constructed by subcloning the fragment from nucleotide -94 to 1766 (Figure 1; i.e., residues 79 to 1938 as presented in SEQ ID NO:1) into the expression vector pGEMHE (Liman, et al., *Neuron*, 9, 861-871, 1992). cRNA was synthesized using the mMessage mMachine transcription kit (Ambion, Austin, TX), with plasmid linearized with *Nhe*I.

The isolation and maintenance of oocytes has been previously described (Boulter, et al., *Proc. Natl. Acad. Sci. USA*, 84, 7763-7767, 1987). Each oocyte was injected with 1 to 10ng of cRNA. Electrophysiological recordings

were performed 2 to 7 days after injection, under two-electrode voltage clamp with an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA). Voltage electrodes were filled with 3M KCl and had a resistance of ~10 MΩ; current electrodes were filled with 0.3M KCl and had a resistance of ~1 MΩ. Unless otherwise stated, the holding potential was -50 mV. I-V relationships were obtained with pClamp 5.5 software (Axon Instruments), by applying 2 second voltage ramps in the presence of agonist and subtracting the control average values obtained before and after agonist application. All records were digitized and stored on a computer. Data was analyzed using software that was designed and provided by Dr. S. Traynelis (The Salk Institute for Biological Studies, La Jolla, CA).

Oocytes were continuously superfused in frog Ringer's solution (10mM HEPES, pH 7.2, 115mM NaCl, 1.8mM $CaCl_2$ and 2.5mM KCl). No responses were observed by the application of drugs to uninjected oocytes. For the inhibition curves (see Figures 4B, 5A and 5B), antagonists were coapplied with 10 μM acetylcholine. In the case of α-bungarotoxin and κ-bungarotoxin (see Figures 6A and 6B), oocytes were preincubated with these drugs for 30 minutes. The mean and standard error of the mean of peak current responses of at least four oocytes per experiment are represented in the Figures. All curve fits were done using Sigma Plot software (Jandel Scientific) with the following equations:

(i) Response (for concentration-response curves) = $[(max-min)/(1+ (EC_{50}/concentration)^n)]+min$, and

(ii) Response (for concentration-inhibition curves) = $[(max-min)/(1+ (concentration/IC_{50})^n)]+min$.

Atropine sulfate, (-)-nicotine ditartrate, (+)-muscarine chloride, strychnine hydrochloride and oxotremorine-M were obtained from RBI (Natick, MA),

kappa-Bungarotoxin was donated by Dr. V. Chiappinelli (St. Louis University Medical Center, St. Louis, MO). All other drugs were obtained from Sigma Chemical Co. (St. Louis, MO). Drugs were dissolved in frog Ringer's solution. Bovine serum albumin (100 mg/ml) was added to the toxin solutions.

EXAMPLE V

In situ Hybridization

Experiments were carried out using mid-sagittal sections of rat E16 embryos (Hybrid-ready tissue, Novagen, Madison, WI) and 20μm thick coronal sections of adult rat brains, according to the protocol described by Simmons, et al. in *J. Histotechnol.*, 12, 169-181, 1989. Either ^{35}S- or ^{32}P-labeled RNA probes were derived from the alpha9 cDNA (e.g., nucleotides 283 to 806, Figure 1; i.e., nucleotides 455 to 979 of SEQ ID NO:1). Hybridization was carried out at 65°C and final washes were carried out at 72°C in 0.1 x SSC (1 x SSC is 180mM NaCl and 17mM sodium citrate, pH 7.0). Slides were dipped in Kodak NTB-2 emulsion, developed in Kodak D19 after 3 weeks exposure at 4°C and subsequently Nissl stained.

EXAMPLE VI

Amplification Reactions

Tissues were obtained from adult Sprague Dawley rats. The animals were decapitated and the tissues were quickly dissected and immersed in liquid nitrogen. Total RNA was isolated according to Chomczynski and Sacchi (see *Analytical Biochem.*, 162, 156-159, 1987), using the TRIzol reagent (Gibco BRL, Gaithersburg, MD). First strand cDNA was synthesized from 2 μg of total RNA with the Superscript Preamplification System (Gibco BRL). An aliquot containing 50ng of cDNA was used as template in amplification reactions. The following specific primers for alpha9 were

employed: sense primer, nucleotides 778 to 802; antisense primer, nucleotide 1353 to 1327 (Figure 1; nucleotides 951 to 975 and nucleotides 1526 to 1500, respectively, of SEQ ID NO:1). The predicted fragment spans one intron-exon boundary. A 573 base pair band is expected in the case of amplification from cDNA, whereas a fragment of ~1450 bp would result from the amplification of contaminant genomic DNA. Reactions were done in the following reaction mixture: 5U of Taq DNA polymerase, 5U of Taq enhancer (Stratagene, La Jolla, CA), 5 μM of each primer, 50 μM each of dATP, dGTP, dCTP and dTTP, 20mM Tris-HCl, pH 8.5, 10mM $(H_4N)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton X-100 and 0.1 mg/ml bovine serum albumin. Cycle parameters were: 2 min. at 95°C followed by 34 cycles each of 1 min. at 55°C, 1 min. at 72°C, 30 sec. at 95°C and a final cycle of 1 min. at 55°C, 5 min. at 72°C.

EXAMPLE VII

Detection of alpha9 Transcripts in Rat Cochlea

To determine if the alpha9 gene is expressed in rat cochlea, amplification reactions were performed on cDNA reverse transcribed from cochlear total RNA. As described in Example V, two primers specific for the alpha9 sequence were employed in order to amplify a fragment that spans an intron-exon boundary and additionally to avoid possible amplification from genomic DNA. Since alpha9 is present in the rat olfactory epithelium, cDNA obtained from this tissue was used as a positive control. Sciatic nerve cDNA was included to rule out the possibility that, with the parameters used for the amplification reactions, very small amounts of transcripts would be detected in any tissue studied. Whereas no DNA was amplified from the sciatic nerve using specific primers for alpha9 (Figure 8), both alpha3 and alpha4 subunits could be detected in this tissue with the respective specific primers.

A fragment of the expected size (573 bp) for amplification from alpha9 cDNA was obtained in the rat cochlea. Restriction endonuclease analysis of the fragment with *Acc*I, *Hinf*I and *Nco*I, further confirmed that this fragment had been derived from alpha9 transcripts.

Although the invention has been described with reference to the specific embodiments, those skilled in the art will readily appreciate that the specific experiments taught hereinabove are only illustrative of the invention. It should be understood that various modifications and variations can be made without departing from the spirit and scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: ELGOYHEN, ANA BELEN
JOHNSON, DAVID S.
BOULTER, JAMES R.
HEINEMANN, STEPHEN F.

(ii) TITLE OF INVENTION: CLONING AND EXPRESSION OF A NOVEL ACETYLCHOLINE-GATED ION CHANNEL RECEPTOR

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: PRETTY, SCHROEDER, BRUEGGEMANN & CLARK
(B) STREET: 444 SOUTH FLOWER STREET, SUITE 2000
(C) CITY: LOS ANGELES
(D) STATE: CALIFORNIA
(E) COUNTRY: USA
(F) ZIP: 90071

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/278,635
(B) FILING DATE: 21-JUL-1994
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: REITER, STEPHEN E.
(B) REGISTRATION NUMBER: 31,192
(C) REFERENCE/DOCKET NUMBER: P41 9771

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 619-546-4737
(B) TELEFAX: 619-546-9392

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1938 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: ALPHA 9

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 89..1525

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
CGGTGGCAGT GAGGGTGTTT TGAGCCCTTC ACAGACAGAA GTGGGAGTCC TCGCTGTCTG      60

CCTGACACAT TCTACATGTT GGGAAAAG ATG AAC CGG CCC CAT TCC TGC CTC         112
                               Met Asn Arg Pro His Ser Cys Leu
                                 1               5

TCC TTT TGC TGG ATG TAT TTT GCT GCT TCT GGA ATC AGA GCC GTA GAG        160
Ser Phe Cys Trp Met Tyr Phe Ala Ala Ser Gly Ile Arg Ala Val Glu
     10                  15                  20

ACA GCA AAT GGG AAA TAT GCT CAG AAA TTG TTC AGC GAT CTT TTT GAA        208
Thr Ala Asn Gly Lys Tyr Ala Gln Lys Leu Phe Ser Asp Leu Phe Glu
 25                  30                  35                  40

GAC TAC TCC AGT GCT CTG CGT CCA GTC GAG GAT ACG GAC GCG GTG CTG        256
Asp Tyr Ser Ser Ala Leu Arg Pro Val Glu Asp Thr Asp Ala Val Leu
                 45                  50                  55

AAT GTT ACA CTG CAG GTC ACG CTC TCC CAG ATA AAG GAC ATG GAC GAG        304
Asn Val Thr Leu Gln Val Thr Leu Ser Gln Ile Lys Asp Met Asp Glu
            60                  65                  70

AGA AAC CAG ATT CTG ACA GCC TAT CTA TGG ATC CGC CAA ACC TGG CAC        352
Arg Asn Gln Ile Leu Thr Ala Tyr Leu Trp Ile Arg Gln Thr Trp His
         75                  80                  85

GAT GCG TAC CTC ACG TGG GAT CGA GAC CAG TAT GAT AGG CTG GAC TCC        400
Asp Ala Tyr Leu Thr Trp Asp Arg Asp Gln Tyr Asp Arg Leu Asp Ser
     90                  95                  100

ATC AGG ATT CCC AGC GAT CTG GTG TGG AGG CCG GAC ATT GTC CTA TAC        448
Ile Arg Ile Pro Ser Asp Leu Val Trp Arg Pro Asp Ile Val Leu Tyr
105                 110                 115                 120

AAC AAG GCT GAC GAT GAG TCT TCA GAG CCT GTG AAC ACC AAT GTG GTG        496
Asn Lys Ala Asp Asp Glu Ser Ser Glu Pro Val Asn Thr Asn Val Val
                125                 130                 135

CTG CGA TAT GAT GGG CTC ATC ACC TGG GAC TCA CCG GCC ATC ACC AAA        544
Leu Arg Tyr Asp Gly Leu Ile Thr Trp Asp Ser Pro Ala Ile Thr Lys
            140                 145                 150

AGC TCC TGT GTG GTG GAT GTC ACC TAC TTC CCT TTT GAC AGC CAG CAG        592
Ser Ser Cys Val Val Asp Val Thr Tyr Phe Pro Phe Asp Ser Gln Gln
        155                 160                 165

TGC AAC CTG ACC TTT GGC TCC TGG ACC TAC AAT GGA AAC CAG GTG GAC        640
Cys Asn Leu Thr Phe Gly Ser Trp Thr Tyr Asn Gly Asn Gln Val Asp
    170                 175                 180

ATA TTC AAT GCC CTG GAC AGC GGT GAC CTC TCT GAC TTC ATT GAA GAT        688
Ile Phe Asn Ala Leu Asp Ser Gly Asp Leu Ser Asp Phe Ile Glu Asp
185                 190                 195                 200

GTG GAA TGG GAG GTC CAT GGC ATG CCT GCT GTA AAG AAC GTC ATC TCC        736
Val Glu Trp Glu Val His Gly Met Pro Ala Val Lys Asn Val Ile Ser
                205                 210                 215

TAT GGC TGC TGC TCC GAG CCT TAC CCA GAT GTC ACC TTC ACT CTC CTT        784
Tyr Gly Cys Cys Ser Glu Pro Tyr Pro Asp Val Thr Phe Thr Leu Leu
            220                 225                 230

CTG AAG AGG AGG TCC TCC TTC TAC ATC GTC AAC CTC CTC ATC CCT TGC        832
Leu Lys Arg Arg Ser Ser Phe Tyr Ile Val Asn Leu Leu Ile Pro Cys
        235                 240                 245
```

```
GTC CTC ATA TCG TTC CTC GCT CCG TTG AGT TTC TAT CTC CCA GCA GCC        880
Val Leu Ile Ser Phe Leu Ala Pro Leu Ser Phe Tyr Leu Pro Ala Ala
    250                 255                 260

TCT GGG GAG AAG GTC TCT CTG GGA GTG ACC ATC CTA TTG GCC ATG ACT        928
Ser Gly Glu Lys Val Ser Leu Gly Val Thr Ile Leu Leu Ala Met Thr
265                 270                 275                 280

GTG TTT CAG CTA ATG GTG GCA GAG ATC ATG CCA GCC TCA GAA AAT GTC        976
Val Phe Gln Leu Met Val Ala Glu Ile Met Pro Ala Ser Glu Asn Val
                285                 290                 295

CCT CTG ATA GGA AAA TAC TAC ATA GCT ACC ATG GCC TTG ATC ACT GCC       1024
Pro Leu Ile Gly Lys Tyr Tyr Ile Ala Thr Met Ala Leu Ile Thr Ala
            300                 305                 310

TCC ACA GCC CTT ACC ATC ATG GTG ATG AAT ATT CAC TTC TGT GGA GCT       1072
Ser Thr Ala Leu Thr Ile Met Val Met Asn Ile His Phe Cys Gly Ala
        315                 320                 325

GAG GCA CGG CCA GTG CCA CAC TGG GCC AAG GTG GTC ATC CTG AAG TAC       1120
Glu Ala Arg Pro Val Pro His Trp Ala Lys Val Val Ile Leu Lys Tyr
    330                 335                 340

ATG TCC AGG ATC TTG TTT GTC TAC GAT GTG GGT GAG AGC TGC CTT AGT       1168
Met Ser Arg Ile Leu Phe Val Tyr Asp Val Gly Glu Ser Cys Leu Ser
345                 350                 355                 360

CCC CGC CAC AGC CAG GAG CCA GAA CAA GTC ACG AAG GTT TAT AGC AAA       1216
Pro Arg His Ser Gln Glu Pro Glu Gln Val Thr Lys Val Tyr Ser Lys
                365                 370                 375

CTC CCA GAA TCC AAC CTG AAA ACG TCC AGA AAC AAA GAC CTT TCC AGA       1264
Leu Pro Glu Ser Asn Leu Lys Thr Ser Arg Asn Lys Asp Leu Ser Arg
            380                 385                 390

AAG AAG GAA GTA AGA AAA CTC TTA AAG AAT GAC CTG GGG TAC CAG GGT       1312
Lys Lys Glu Val Arg Lys Leu Leu Lys Asn Asp Leu Gly Tyr Gln Gly
        395                 400                 405

GGG ATC CCC CAG AAT ACT GAC AGT TAT TGT GCA CGC TAT GAA GCA CTG       1360
Gly Ile Pro Gln Asn Thr Asp Ser Tyr Cys Ala Arg Tyr Glu Ala Leu
    410                 415                 420

GCG AAA AAT ATC GAA TAC ATT GCC AAG TGC CTC AAG GAC CAC AAG GCC       1408
Ala Lys Asn Ile Glu Tyr Ile Ala Lys Cys Leu Lys Asp His Lys Ala
425                 430                 435                 440

ACC AAC TCC AAG GGC AGC GAG TGG AAG AAG GTC GCC AAA GTC ATA GAC       1456
Thr Asn Ser Lys Gly Ser Glu Trp Lys Lys Val Ala Lys Val Ile Asp
                445                 450                 455

CGT TTC TTC ATG TGG ATT TTC TTT GCT ATG GTG TTT GTC ATG ACC GTC       1504
Arg Phe Phe Met Trp Ile Phe Phe Ala Met Val Phe Val Met Thr Val
            460                 465                 470

TTG ATC ATA GCA AGA GCA GAT TAGCAGGAAA GAGGAGTGGG CTGGTAGGCA          1555
Leu Ile Ile Ala Arg Ala Asp
        475

TTTAGAGATT TGGGGAAAAC CCAATAAAAT CACCTGAGAT CTGCCCCAGC GTGTGAGTTC     1615

AGCTGCTGTT CATACATAAT TTAGGGGATA GGTTGCGTAT GCTTTTATTC CTAACTTCAA     1675

TCAATATCCT AGTTACATGT CAGGTTAAAT CAAGCAGGAG ATGCAAGGTT TCAAGGGTAA     1735

AGGGCTGGAG GAAGAGAGTT AGAAAGGACC CTTTCACAGG CTCCCATGAA GGGGAGTGGT     1795
```

GGCCTTCAGT TTATGTAATT ATCTCTTTAT TATTGTAGAC AACAAAGCAC AGTGTATTCC 1855

TGCTTAAGAT TTAAAGCAAG AAAAGACAAA ACAAATTCAT TCTCTTAGTC CTTAATAAAA 1915

CTTTTTTTTT TAAACAAAAA AAA 1938

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 479 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Asn Arg Pro His Ser Cys Leu Ser Phe Cys Trp Met Tyr Phe Ala
  1               5                  10                  15

Ala Ser Gly Ile Arg Ala Val Glu Thr Ala Asn Gly Lys Tyr Ala Gln
            20                  25                  30

Lys Leu Phe Ser Asp Leu Phe Glu Asp Tyr Ser Ser Ala Leu Arg Pro
        35                  40                  45

Val Glu Asp Thr Asp Ala Val Leu Asn Val Thr Leu Gln Val Thr Leu
    50                  55                  60

Ser Gln Ile Lys Asp Met Asp Glu Arg Asn Gln Ile Leu Thr Ala Tyr
65                  70                  75                  80

Leu Trp Ile Arg Gln Thr Trp His Asp Ala Tyr Leu Thr Trp Asp Arg
                85                  90                  95

Asp Gln Tyr Asp Arg Leu Asp Ser Ile Arg Ile Pro Ser Asp Leu Val
            100                 105                 110

Trp Arg Pro Asp Ile Val Leu Tyr Asn Lys Ala Asp Asp Glu Ser Ser
        115                 120                 125

Glu Pro Val Asn Thr Asn Val Val Leu Arg Tyr Asp Gly Leu Ile Thr
    130                 135                 140

Trp Asp Ser Pro Ala Ile Thr Lys Ser Ser Cys Val Val Asp Val Thr
145                 150                 155                 160

Tyr Phe Pro Phe Asp Ser Gln Gln Cys Asn Leu Thr Phe Gly Ser Trp
                165                 170                 175

Thr Tyr Asn Gly Asn Gln Val Asp Ile Phe Asn Ala Leu Asp Ser Gly
            180                 185                 190

Asp Leu Ser Asp Phe Ile Glu Asp Val Glu Trp Glu Val His Gly Met
        195                 200                 205

Pro Ala Val Lys Asn Val Ile Ser Tyr Gly Cys Cys Ser Glu Pro Tyr
    210                 215                 220

Pro Asp Val Thr Phe Thr Leu Leu Leu Lys Arg Arg Ser Ser Phe Tyr
225                 230                 235                 240

Ile Val Asn Leu Leu Ile Pro Cys Val Leu Ile Ser Phe Leu Ala Pro
                245                 250                 255
```

Leu Ser Phe Tyr Leu Pro Ala Ala Ser Gly Glu Lys Val Ser Leu Gly
260 265 270

Val Thr Ile Leu Leu Ala Met Thr Val Phe Gln Leu Met Val Ala Glu
275 280 285

Ile Met Pro Ala Ser Glu Asn Val Pro Leu Ile Gly Lys Tyr Tyr Ile
290 295 300

Ala Thr Met Ala Leu Ile Thr Ala Ser Thr Ala Leu Thr Ile Met Val
305 310 315 320

Met Asn Ile His Phe Cys Gly Ala Glu Ala Arg Pro Val Pro His Trp
325 330 335

Ala Lys Val Val Ile Leu Lys Tyr Met Ser Arg Ile Leu Phe Val Tyr
340 345 350

Asp Val Gly Glu Ser Cys Leu Ser Pro Arg His Ser Gln Glu Pro Glu
355 360 365

Gln Val Thr Lys Val Tyr Ser Lys Leu Pro Glu Ser Asn Leu Lys Thr
370 375 380

Ser Arg Asn Lys Asp Leu Ser Arg Lys Lys Glu Val Arg Lys Leu Leu
385 390 395 400

Lys Asn Asp Leu Gly Tyr Gln Gly Gly Ile Pro Gln Asn Thr Asp Ser
405 410 415

Tyr Cys Ala Arg Tyr Glu Ala Leu Ala Lys Asn Ile Glu Tyr Ile Ala
420 425 430

Lys Cys Leu Lys Asp His Lys Ala Thr Asn Ser Lys Gly Ser Glu Trp
435 440 445

Lys Lys Val Ala Lys Val Ile Asp Arg Phe Phe Met Trp Ile Phe Phe
450 455 460

Ala Met Val Phe Val Met Thr Val Leu Ile Ile Ala Arg Ala Asp
465 470 475

What is claimed is:

1. Isolated nucleic acid encoding an alpha9 nicotinic acetylcholine receptor (nAChR) subunit.

2. Isolated nucleic acid according to Claim 1, wherein said nucleic acid comprises DNA.

3. DNA according to Claim 2, wherein said DNA is a cDNA.

4. DNA according to Claim 2, wherein said DNA encodes the amino acid sequence set forth in SEQ ID NO:2.

5. DNA according to Claim 2, wherein said DNA hybridizes under high stringency conditions to the nucleotide sequence set forth in SEQ ID NO:1.

6. DNA according to Claim 2, wherein the nucleotide sequence of said DNA is substantially the same as the nucleotide sequence set forth in SEQ ID NO:1.

7. A vector comprising DNA according to Claim 2.

8. A host cell containing a vector according to Claim 7.

9. A host cell according to Claim 8, wherein said cell expresses functional nACh receptor comprising at least one alpha9 nAChR subunit.

10. A host cell according to Claim 9, wherein said receptor is homomeric.

11. A host cell according to Claim 9, wherein said receptor is heteromeric.

12. A nucleic acid probe comprising at least 14 contiguous nucleotides according to Claim 2.

13. Isolated mRNA complementary to DNA according to Claim 2.

14. An antisense oligonucleotide that specifically binds to and modulates translation of mRNA according to Claim 13.

15. Isolated alpha9 nicotinic acetylcholine receptor subunit, and biologically active fragments thereof.

16. Isolated alpha9 nicotinic acetylcholine receptor subunit according to Claim 15 wherein said subunit has the same amino acid sequence set forth in SEQ ID NO:2.

17. Isolated alpha9 nicotinic acetylcholine receptor subunit according to Claim 15 wherein said subunit has substantially the same amino acid sequence as that set forth in SEQ ID NO:2.

18. A functional nicotinic acetylcholine receptor expressed recombinantly in a host cell, said receptor characterized by:

(i) being expressed in the pituitary, olfactory epithelium, cochlea and tongue;

(ii) being activated by acetylcholine but blocked by nicotine and muscarine, and

(iii) comprising at least one alpha9 acetylcholine receptor subunit.

19. A receptor according to Claim 18 wherein said alpha9 nicotinic acetylcholine receptor subunit has the same amino acid sequence set forth in SEQ ID NO:2.

20. A receptor according to Claim 18 wherein said alpha9 nicotinic acetylcholine receptor subunit has substantially the same amino acid sequence as that set forth in SEQ ID NO:2.

21. An antibody that specifically binds to an alpha9 nicotinic acetylcholine receptor subunit.

22. An antibody according to Claim 21, wherein said antibody is a monoclonal antibody.

23. An antibody according to Claim 21, wherein said antibody is a polyclonal antibody.

24. A composition comprising:

an amount of an oligonucleotide according to Claim 14 effective to modulate expression of a nicotinic acetylcholine receptor comprising at least one alpha9 subunit, and

an acceptable hydrophobic carrier capable of passing through a cell membrane.

25. A composition comprising an amount of the antibody according to Claim 21 effective to block binding of naturally occurring ligands to a nicotinic acetylcholine receptor comprising at least one alpha9 subunit and an acceptable carrier.

26. A transgenic non-human mammal expressing DNA encoding an alpha9 nicotinic acetylcholine receptor subunit.

27. A transgenic non-human mammal according to Claim 26, wherein DNA encoding said receptor has been mutated such that the expressed receptor does not contain wild-type alpha9 nicotinic acetylcholine receptor subunits.

28. A transgenic non-human mammal according to Claim 26, wherein the transgenic non-human mammal is a mouse.

29. A method for identifying nucleic acids encoding alpha9 nicotinic acetylcholine receptor subunit(s), said method comprising:

contacting a sample containing a population of nucleic acids with a probe according to Claim 12, wherein said contacting is effected under high stringency hybridization conditions, and

identifying nucleic acids which hybridize to said probe.

30. A method for identifying compounds which bind to alpha9 nicotinic acetylcholine receptor subunit(s), said method comprising contacting cells according to Claim 9 with test compound and identifying compounds which bind thereto.

31. A bioassay for identifying compounds which modulate the activity of nicotinic acetylcholine receptors comprising at least one alpha9 subunit, said method comprising:

contacting cells according to Claim 9 with at least one compound wherein the ability of said compound to modulate the ion channel of the receptor is unknown; and thereafter

monitoring the cells for changes in ion channel activity.

32. A method according to Claim 31 wherein said cells are contacted with at least one potential agonist.

33. A method according to Claim 31 wherein said cells are contacted with a constant amount of agonist and increasing amounts of at least one potential antagonist.

34. A bioassay for identifying compounds which modulate the regulatory regions(s) of the alpha9 nicotinic acetylcholine receptor subunit gene, said method comprising:

transforming mammalian cells with a nucleic acid construct comprising at least a portion of the regulatory region of the alpha9 gene, wherein said portion of the regulatory gene is operatively associated with a reporter gene;

contacting said transformed cells with at least one compound wherein the ability of the compound to modulate the regulatory region of the alpha9 receptor subunit gene is unknown; and

monitoring said cells for expression of said reporter gene.

```
-153

-120  ACA GAA GTG GGA GTC CTC GCT GTC TGC CTG ACA CAT TCT
 -22


   1  GTA GAG ACA GCA AAT GGG AAA TAT GCT CAG AAA TTG TTC
   1  Val Glu Thr Ala Asn Gly Lys Tyr Ala Gln Lys Leu Phe

 121  ACG CTC TCC CAG ATA AAG GAC ATG GAC GAG AGA AAC CAG
  41  Thr Leu Ser Gln Ile Lys Asp Met Asp Glu Arg Asn Gln

 241  GAC TCC ATC AGG ATT CCC AGC GAT CTG GTG TGG AGG CCG
  81  Asp Ser Ile Arg Ile Pro Ser Asp Leu Val Trp Arg Pro

 361  ATC ACC TGG GAC TCA CCG GCC ATC ACC AAA AGC TCC TGT
 121  Ile Thr Trp Asp Ser Pro Ala Ile Thr Lys Ser Ser Cys

 481  GTG GAC ATA TTC AAT GCC CTG GAC AGC GGT GAC CTC TCT
 161  Val Asp Ile Phe Asn Ala Leu Asp Ser Gly Asp Leu Ser

 601  CCT TAC CCA GAT GTC ACC TTC ACT CTC CTT CTG AAG AGG
 201  Pro Tyr Pro Asp Val Thr Phe Thr Leu Leu Leu Lys Arg


 721  GCA GCC TCT GGG GAG AAG GTC TCT CTG GGA GTG ACC ATC
 241  Ala Ala Ser Gly Glu Lys Val Ser Leu Gly Val Thr Ile
      -------                      ----------------------

 841  TAC ATA GCT ACC ATG GCC TTG ATC ACT GCC TCC ACA GCC
 281  Tyr Ile Ala Thr Met Ala Leu Ile Thr Ala Ser Thr Ala
      ----------------------------MSR III----------------

 961  AAG TAC ATG TCC AGG ATC TTG TTT GTC TAC GAT GTG GGT
 321  Lys Tyr Met Ser Arg Ile Leu Phe Val Tyr Asp Val Gly

1081  AAA ACG TCC AGA AAC AAA GAC CTT TCC AGA AAG AAG GAA
 361  Lys Thr Ser Arg Asn Lys Asp Leu Ser Arg Lys Lys Glu

1201  GCA CTG GCG AAA AAT ATC GAA TAC ATT GCC AAG TGC CTC
 401  Ala Leu Ala Lys Asn Ile Glu Tyr Ile Ala Lys Cys Leu


1321  TTC TTT GCT ATG GTG TTT GTC ATG ACC GTC TTG ATC ATA
 441  Phe Phe Ala Met Val Phe Val Met Thr Val Leu Ile Ile
      --MSR IV-------------------------------------------

1441  TCT GCC CCA GCG TGT GAG TTC AGC TGC TGT TCA TAC ATA
1561  GAT GCA AGG TTT CAA GGG TAA AGG GCT GGA GGA AGA GAG
1681  CAA CAA AGC ACA GTG TAT TCC TGC TTA AGA TTT AAA GCA
```

FIG. 1A

```
ACA TGT TGG GAA AAG ATG AAC CGG CCC CAT TCC TGC CTC TCC
                    Met Asn Arg Pro His Ser Cys Leu Ser
                    ----------------------------------

AGC GAT CTT TTT GAA GAC TAC TCC AGT GCT CTG CGT CCA GTC
Ser Asp Leu Phe Glu Asp Tyr Ser Ser Ala Leu Arg Pro Val

ATT CTG ACA GCC TAT CTA TGG ATC CGC CAA ACC TGG CAC GAT
Ile Leu Thr Ala Tyr Leu Trp Ile Arg Gln Thr Trp His Asp
                           ↓
GAC ATT GTC CTA TAC AAC AAG GCT GAC GAT GAG TCT TCA GAG
Asp Ile Val Leu Tyr Asn Lys Ala Asp Asp Glu Ser Ser Glu

GTG GTG GAT GTC ACC TAC TTC CCT TTT GAC AGC CAG CAG TGC
Val Val Asp Val Thr Tyr Phe Pro Phe Asp Ser Gln Gln Cys

GAC TTC ATT GAA GAT GTG GAA TGG GAG GTC CAT GGC ATG CCT
Asp Phe Ile Glu Asp Val Glu Trp Glu Val His Gly Met Pro

AGG TCC TCC TTC TAC ATC GTC AAC CTC CTC ATC CCT TGC GTC
Arg Ser Ser Phe Tyr Ile Val Asn Leu Leu Ile Pro Cys Val
    ------------------------------------------------

CTA TTG GCC ATG ACT GTG TTT CAG CTA ATG GTG GCA GAG ATC
Leu Leu Ala Met Thr Val Phe Gln Leu Met Val Ala Glu Ile
-----------------MSR II---------------------------------

CTT ACC ATC ATG GTG ATG AAT ATT CAC TTC TGT GGA GCT GAG
Leu Thr Ile Met Val Met Asn Ile His Phe Cys Gly Ala Glu
----------------------------

GAG AGC TGC CTT AGT CCC CGC CAC AGC CAG GAG CCA GAA CAA
Glu Ser Cys Leu Ser Pro Arg His Ser Gln Glu Pro Glu Gln

GTA AGA AAA CTC TTA AAG AAT GAC CTG GGG TAC CAG GGT GGG
Val Arg Lys Leu Leu Lys Asn Asp Leu Gly Tyr Gln Gly Gly

AAG GAC CAC AAG GCC ACC AAC TCC AAG GGC AGC GAG TGG AAG
Lys Asp His Lys Ala Thr Asn Ser Lys Gly Ser Glu Trp Lys


GCA AGA GCA GAT TAG CAG GAA AGA GGA GTG GGC TGG TAG GCA
Ala Arg Ala Asp
---------------

ATT TAG GGG ATA GGT TGC GTA TGC TTT TAT TCC TAA CTT CAA
TTA GAA AGG ACC CTT TCA CAG GCT CCC ATG AAG GGG AGT GGT
AGA AAA GAC AAA ACA AAT TCA TTC TCT TAG TCC TTA ATA AAA
```

FIG. 1B

```
        GGT GGC AGT GAG GGT GTT TTG AGC CCT TCA CAG  -121
                                                 ↓
TTT TGC TGG ATG TAT TTT GCT GCT TCT GGA ATC AGA GCC    -1
Phe Cys Trp Met Tyr Phe Ala Ala Ser Gly Ile Arg Ala    -1
-SIGNAL PEPTIDE-------------------------------------

GAG GAT ACG GAC GCG GTG CTG AAT GTT ACA CTG CAG GTC   120
Glu Asp Thr Asp Ala Val Leu Asn Val Thr Leu Gln Val    40

GCG TAC CTC ACG TGG GAT CGA GAC CAG TAT GAT AGG CTG   240
Ala Tyr Leu Thr Trp Asp Arg Asp Gln Tyr Asp Arg Leu    80

CCT GTG AAC ACC AAT GTG GTG CTG CGA TAT GAT GGG CTC   360
Pro Val Asn Thr Asn Val Val Leu Arg Tyr Asp Gly Leu   120

AAC CTG ACC TTT GGC TCC TGG ACC TAC AAT GGA AAC CAG   480
Asn Leu Thr Phe Gly Ser Trp Thr Tyr Asn Gly Asn Gln   160

GCT GTA AAG AAC GTC ATC TCC TAT GGC TGC TGC TCC GAG   600
Ala Val Lys Asn Val Ile Ser Tyr Gly Cys Cys Ser Glu   200

CTC ATA TCG TTC CTC GCT CCG TTG AGT TTC TAT CTC CCA   720
Leu Ile Ser Phe Leu Ala Pro Leu Ser Phe Tyr Leu Pro   240
----------------MSR I-------------------------------
                                          ↓
ATG CCA GCC TCA GAA AAT GTC CCT CTG ATA GGA AAA TAC   840
Met Pro Ala Ser Glu Asn Val Pro Leu Ile Gly Lys Tyr   280
--------                    ----------------------

GCA CGG CCA GTG CCA CAC TGG GCC AAG GTG GTC ATC CTG   960
Ala Arg Pro Val Pro His Trp Ala Lys Val Val Ile Leu   320

GTC ACG AAG GTT TAT AGC AAA CTC CCA GAA TCC AAC CTG  1080
Val Thr Lys Val Tyr Ser Lys Leu Pro Glu Ser Asn Leu   360

ATC CCC CAG AAT ACT GAC AGT TAT TGT GCA CGC TAT GAA  1200
Ile Pro Gln Asn Thr Asp Ser Tyr Cys Ala Arg Tyr Glu   400

AAG GTC GCC AAA GTC ATA GAC CGT TTC TTC ATG TGG ATT  1320
Lys Val Ala Lys Val Ile Asp Arg Phe Phe Met Trp Ile   440
-----------------------------------------------------

TTT AGA GAT TTG GGG AAA ACC CAA TAA AAT CAC CTG AGA  1440
                                                     457


TCA ATA TCC TAG TTA CAT GTC AGG TTA AAT CAA GCA GGA  1560
GGC CTT CAG TTT ATG TAA TTA TCT CTT TAT TAT TGT AGA  1680
CTT TTT TTT TTA AAC AAA AAA AA                      1784
```

FIG. 1C

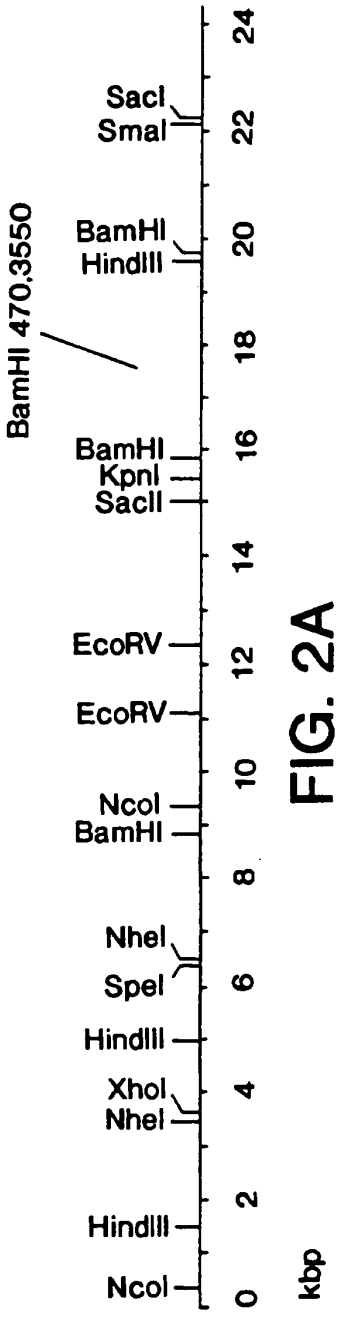
BamHI 470,3550
SacI
SmaI
BamHI
HindIII
BamHI
KpnI
SacII
EcoRV
EcoRV
NcoI
BamHI
NheI
SpeI
HindIII
XhoI
NheI
HindIII
NcoI
0
2
4
6
8
10
12
14
16
18
20
22
24
kbp

FIG. 2A

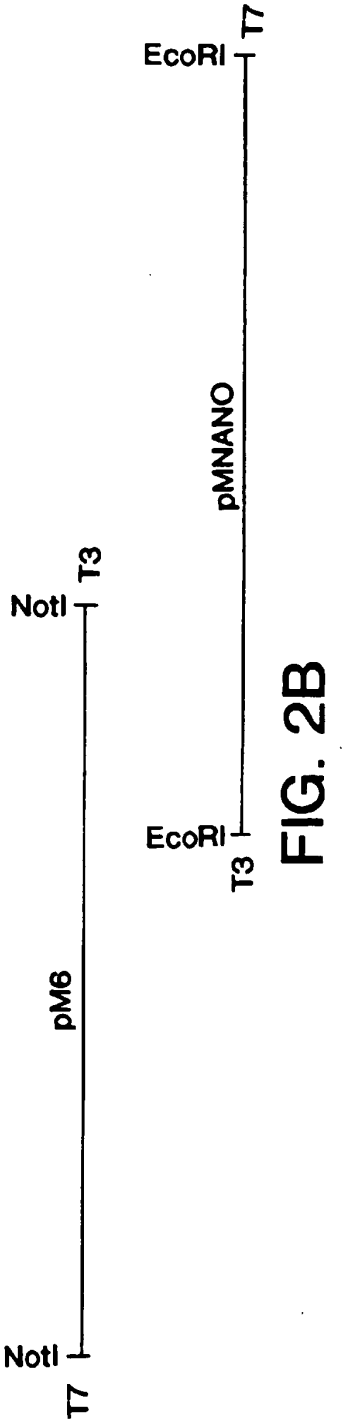
T7
EcoRI
pMNANO
EcoRI
T3
T3
NotI
pM6
NotI
T7

FIG. 2B

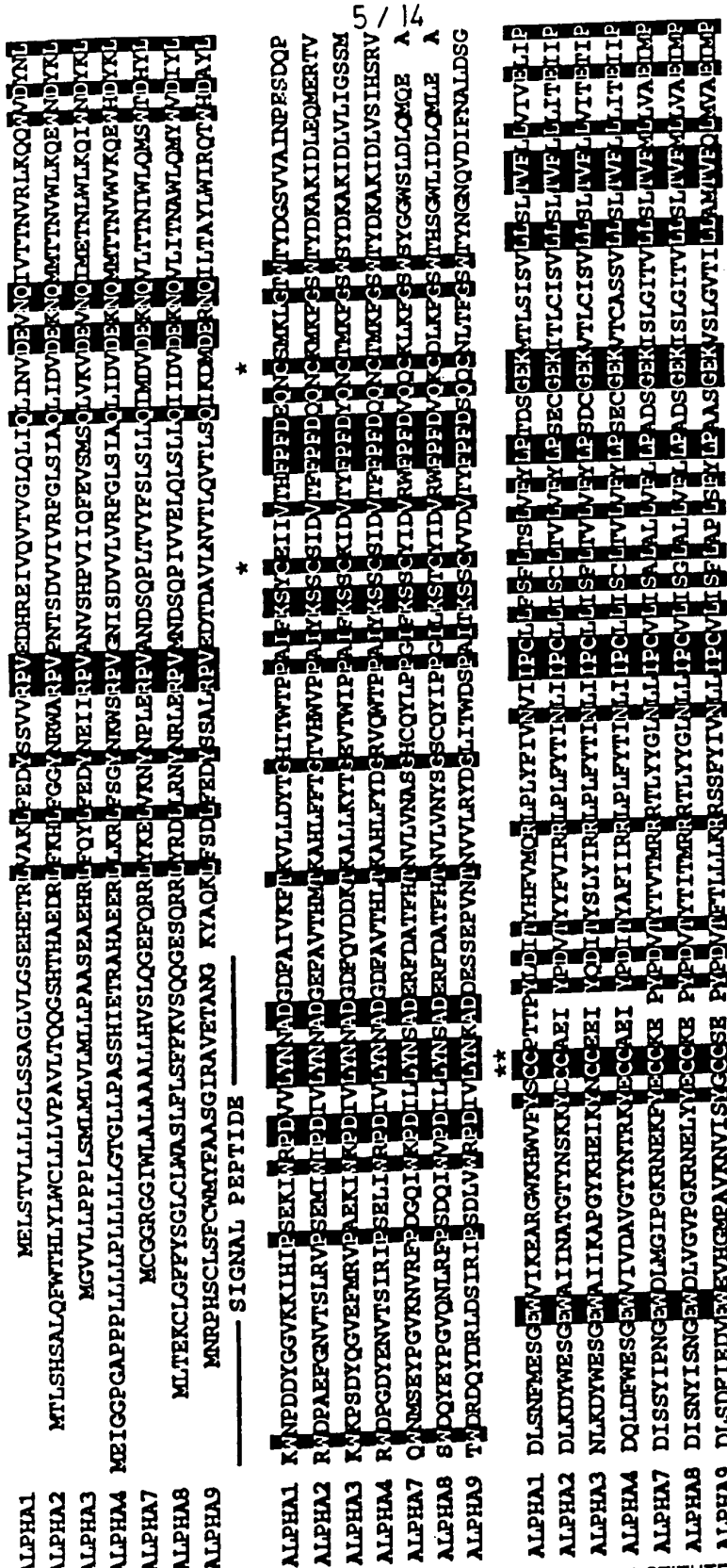
ALPHA1
ALPHA2
ALPHA3
ALPHA4
ALPHA7
ALPHA8
ALPHA9
SIGNAL PEPTIDE
MSR I
MSR II

FIG. 3A

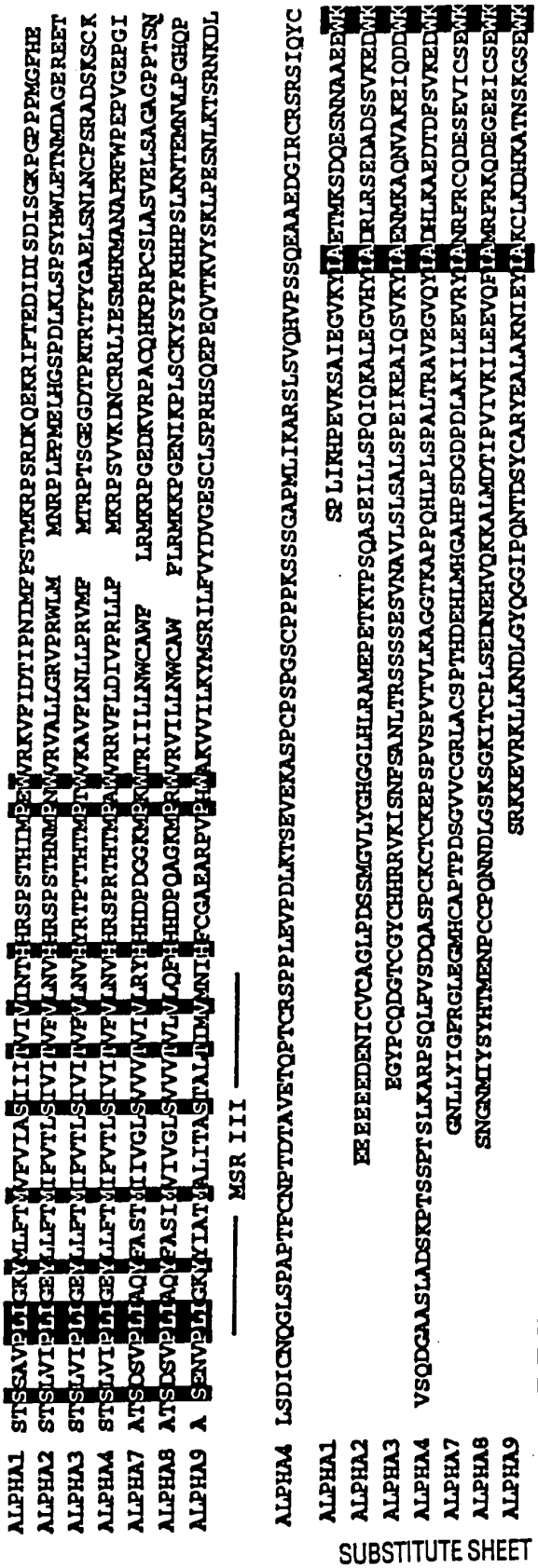

FIG. 3B

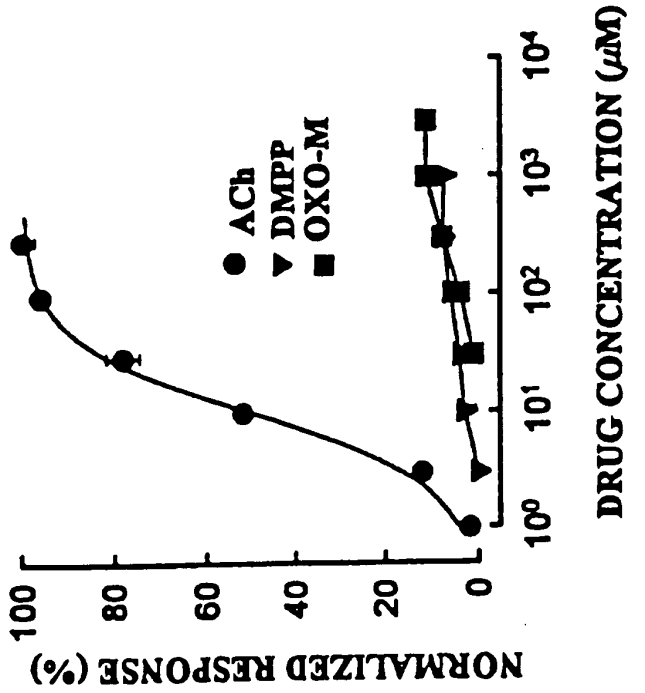
NORMALIZED RESPONSE (%)
100
80
60
40
20
0
ACh
DMPP
OXO-M
10^0
10^1
10^2
10^3
10^4
DRUG CONCENTRATION (μM)

FIG. 4B

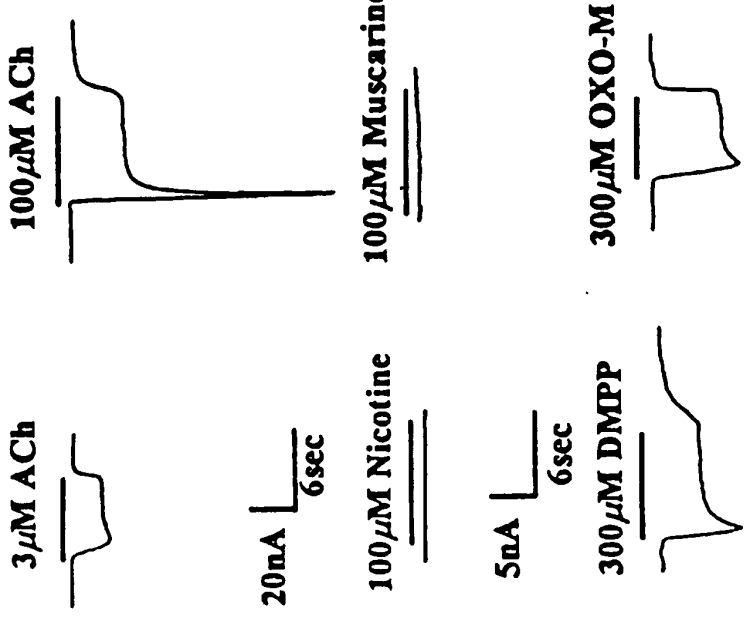
3μM ACh
20nA
6sec
100μM ACh
100μM Nicotine
5nA
6sec
100μM Muscarine
300μM DMPP
5nA
6sec
300μM OXO-M

FIG. 4A

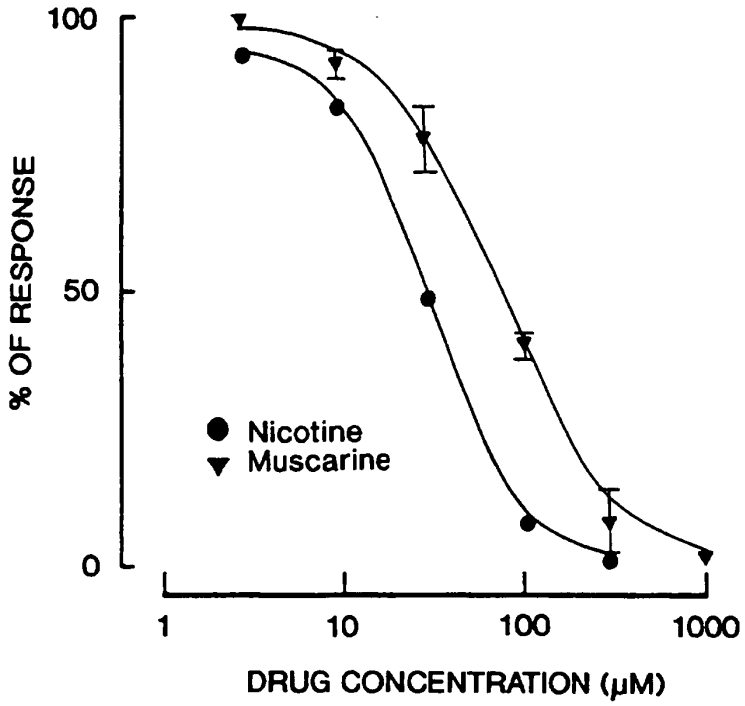
100
50
0
% OF RESPONSE
Nicotine
Muscarine
1
10
100
1000
DRUG CONCENTRATION (μM)

FIG. 5A

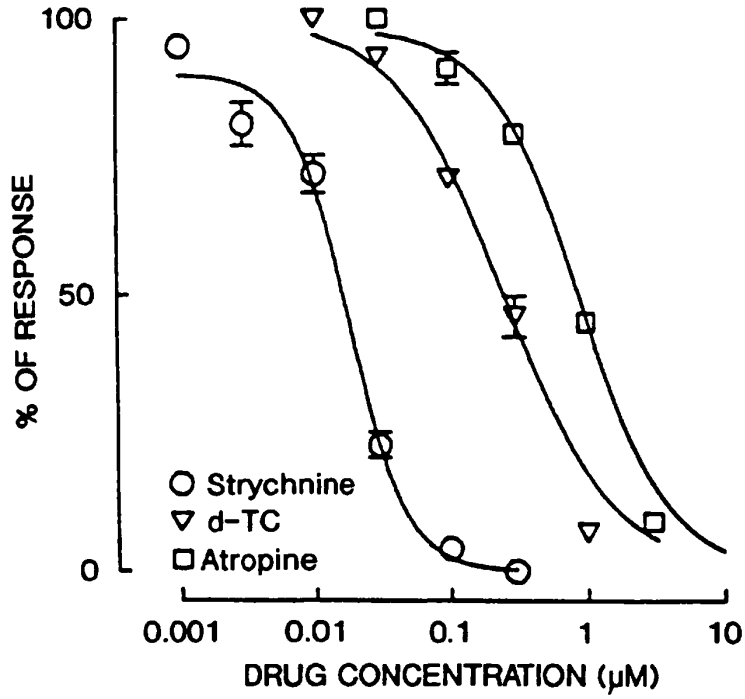
100
50
0
% OF RESPONSE
Strychnine
d-TC
Atropine
0.001
0.01
0.1
1
10
DRUG CONCENTRATION (μM)

FIG. 5B

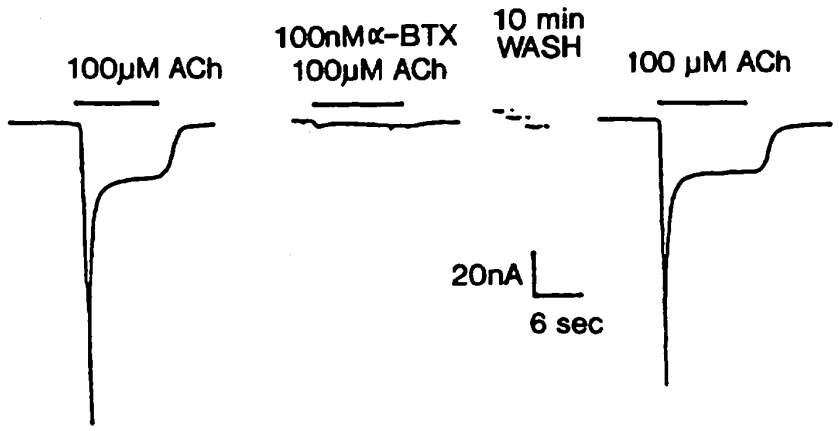
100μM ACh
100nMα-BTX
100μM ACh
10 min
WASH
100 μM ACh
20nA
6 sec

FIG. 6A

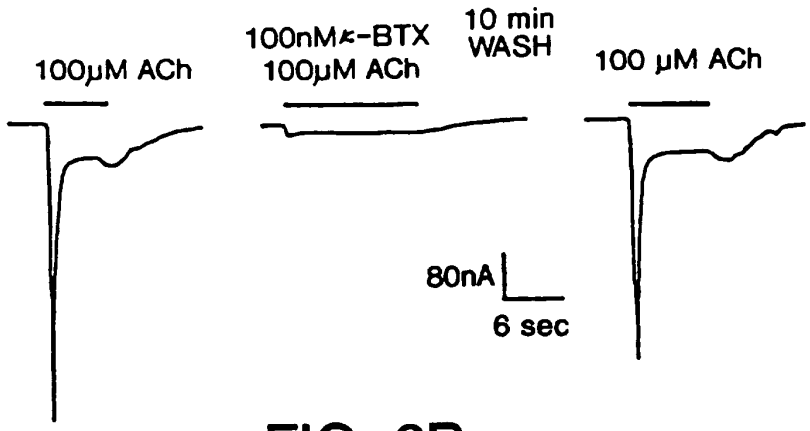
100μM ACh
100nMα-BTX
100μM ACh
10 min
WASH
100 μM ACh
80nA
6 sec

FIG. 6B

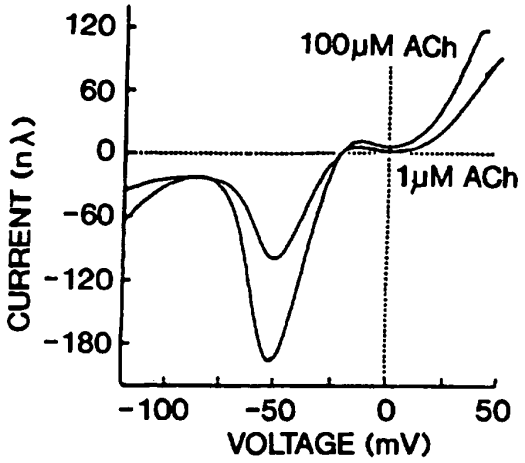

FIG. 7A

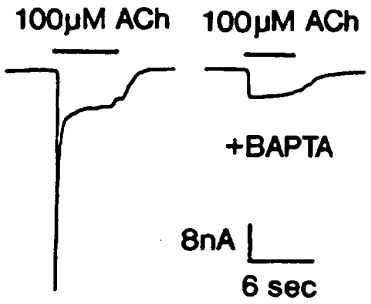

FIG. 7B

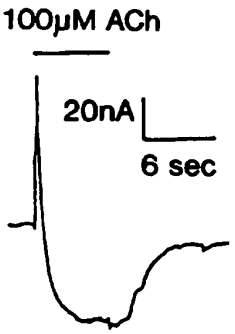

FIG. 7C

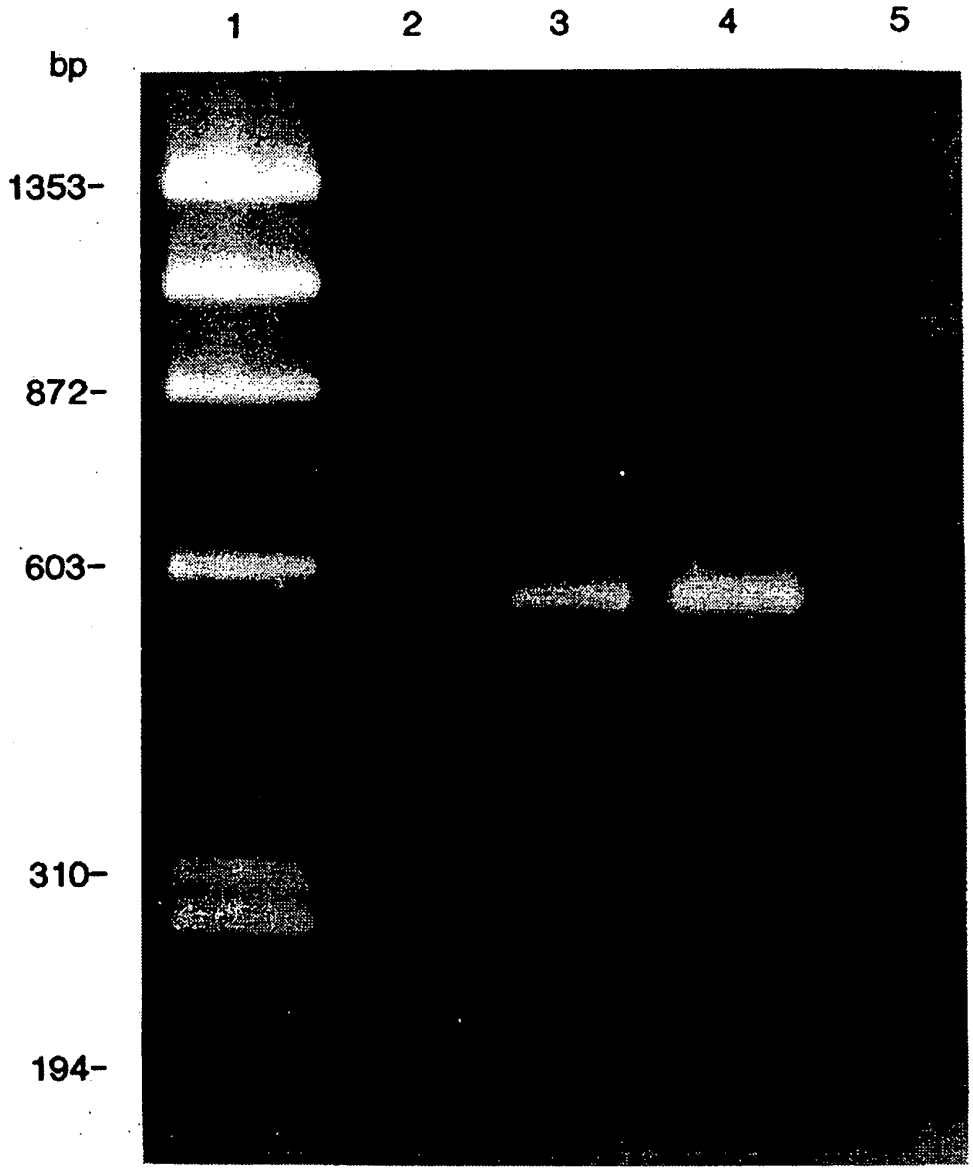

FIG. 8

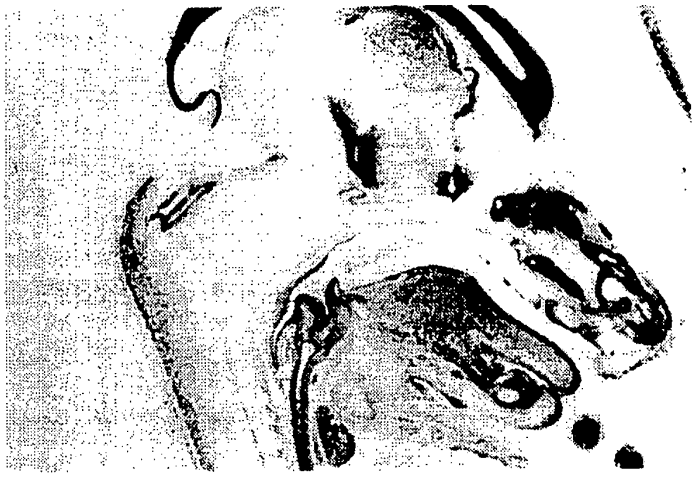

FIG. 9A

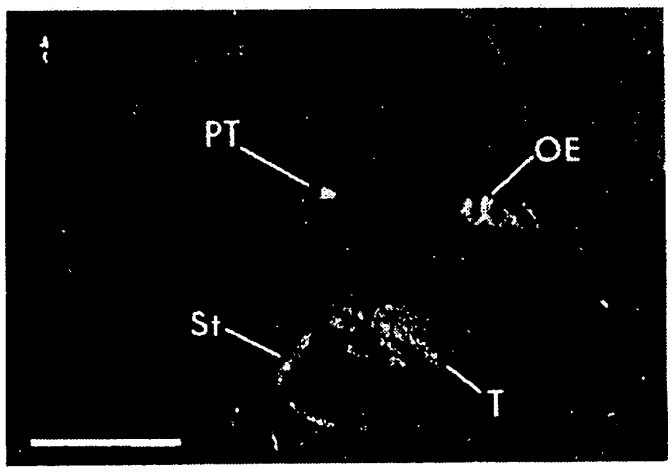

FIG. 9B

FIG. 9C

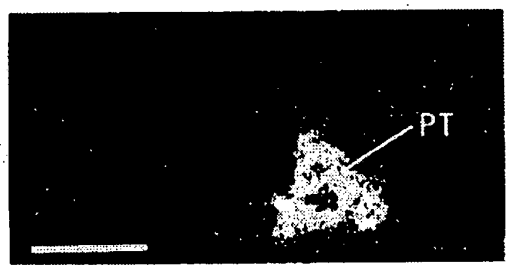

FIG. 9D

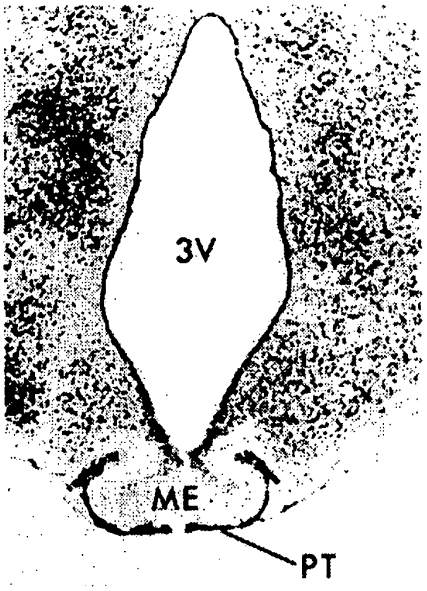

FIG. 9E

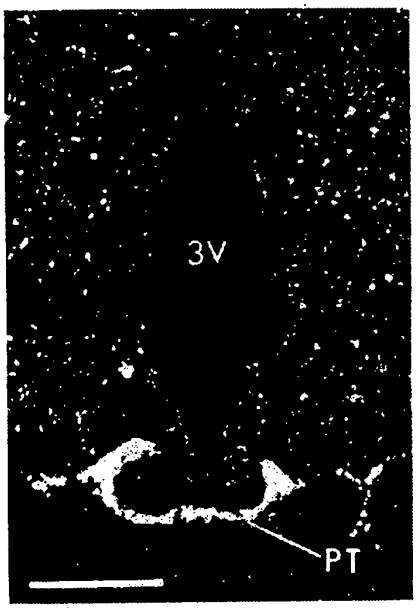

FIG. 9F

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/09104

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N15/85 C07K14/705 C12N5/10 C12Q1/68
C07K16/28 A01K67/027 C12Q1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N C12Q A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,91 15602 (THE SALK INSTITUTE BIOTECHNOLOGY/INDUSTRIAL ASSOCIATES, INC.) 17 October 1991 see page 5, line 5 - page 6, line 27 see page 8, line 5 - page 12, line 15 see page 13, line 34 - page 18, line 27 --- -/--	1-34

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 December 1995	28.12.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Montero Lopez, B

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/09104

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	CELL (CAMBRIDGE, MASS.) (1994), 79(4), 705-15 CODEN: CELLB5;ISSN: 0092-8674, 1994 ELGOYHEN, ANA B. ET AL 'Acetylcholine receptor. alpha. 9 with novel pharmacological properties expressed in rat cochlear hair cells' see the summary see page 705, right column, paragraph 4 - page 706, left column, paragraph 1 see page 711, left column, paragraph 1 see page 713, left column, paragraph 7 -----	1-9,12, 29

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCI/US 95/09104

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9115602	17-10-91	US-A-	5369028	29-11-94
		EP-A-	0523187	20-01-93